NANOPARTICLE-MEDIATED TRANSCRIPTIONAL REGULATION OF STEM AND PROGENITOR CELLS FOR PERIPHERAL NERVE REGENERATION

by
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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

October, 2016

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Abstract

Schwann cells offer great promise in treating peripheral nerve injuries as they can assist in clearing debris, provide trophic support, and importantly, remyelinate regenerating axons, but there are no readily available sources of primary Schwann cells for peripheral nerve cell therapies. While Schwann cell-like cells have been derived from *e.g.* embryonic stem cells, mesenchymal stem cells and skin-derived precursors, these cells have yet to show significant myelination activity compared to primary Schwann cells. This is the main hurdle for their utility in cell-based nerve regenerative therapies. Cell reprogramming has opened up the possibility to derive Schwann cell progenitors from accessible sources. A critical challenge however remains in achieving high functionality in these cells. The use of transcription factor overexpression, typically achieved using viral vectors, is a promising method of directing cell fate and enhancing cell maturation. However, risks associated with viral vectors limit their utility in future clinical applications. In my thesis dissertation, a polymer-based nanoparticle transfection method for overexpression of transcription factors and other genes of interest in human pluripotent stem cell-derived Schwann cell precursors was developed. After screening candidate transcription factors, overexpression of *Egr2* was shown to enhance the myelination frequency of primary rat Schwann cells. To expand the cell types for supporting peripheral nerve regeneration, oligodendrocyte progenitor cells found in the central nervous system were shown to survive and function in a peripheral nerve injury model. These results demonstrated for the first time that oligodendrocyte progenitor cells survive heterotopic engraftment to the peripheral nervous system, and transdifferentiate into a Schwann cell-like phenotype capable of remyelinating regenerating axons. thus

supporting the oligodendrocyte progenitor cells as a new candidate cell type for regenerative therapies in the peripheral nervous system.

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Acknowledgements

I am a strong believer in that an inquisitive mind must be nurtured from an early age to reach fruition, and therefore wish to first thank all my mentors from my early years. This includes first and foremost my parents and grandparents, who have led by both example and encouragement, as well as allowed me to explore any interests I have had without ever pressuring me regarding my choices. The only demand I have ever felt is that I am serious about what I do, and that I go about it in a responsible way seeing through my commitments. I would also like to thank Rasmus, my brother. While he is almost seven years younger than me, he is much wiser and more responsible than me. He is currently in his senior year of a M.Sc. program in Applied Physics and Electrical Engineering, specializing in mechatronics, and is also a very skilled classical guitarist. The things he and his classmates do already at this age just blow my mind, and I have always been proud to get to call him my brother.

A few key teachers, who let me explore my interests in the sciences, especially when they were outside the curriculum, played a key part in shaping my early interest. These people are Torvald Segerstedt, my science and math teacher in grades 7 through 9, and my most inspirational high school science teachers Per Lundmark, Per Sjöström, and Jonas Sjunnesson. These people went above and beyond what was asked from them as teachers. Torvald saw to it that I got to work on problems that I found more challenging than the normal curriculum, despite it being extra work for him, and made sure that I had a safe space to explore in a class that had all kinds of problems and distractions. My high school teachers always taught with passion, which is important to keep young minds

interested. Per Lundmark created special classrooms equipped with all kinds of experiments for students to explore and spark their interest in the sciences, Per Sjöström was a trained marine biologist and took us out on field trips to learn biology though field studies, not stopping even though local law enforcement was alerted when his students were throwing chunks of potassium into the river, and Jonas Sjunnesson who loved physics so much he spent his evenings reading research papers, working on his textbooks, and I believe he even named his son Newton.

I would also not have been here if it wasn't for my mentor during my M.Sc. thesis project, Dr. Joanne Hackett. In the short period of time that I worked with her, she started refining my scientific thinking and writing at a great pace. She saw that I thrived under freedom, and said that whatever ideas I had, I was welcome to explore. At this point I had never seen anyone with such faith and trust in me, especially with the very limited experience that I had prior to that. Her door was always open, and some days we would meet up to ten times in a day, and she was always patient with my questions big or small. She was also instrumental in showing me how to promote myself, got me connected to all of my graduate school interviews, and was instrumental through her support and recommendations to help me secure my fellowships throughout the years I have been at Hopkins. She has moved on from her job at Linköping University, and is currently doing phenomenally at Cambridge University. She remains a close friend of mine, and is a person for whom I have the utmost admiration.

My academic career would have been cut short if it wasn't for Dr. Elvar Theodorsson, a professor in neuroscience who made space for me in his lab when a

conflict prevented me from continuing my thesis work in my original lab space at Linköping. After completing my M.Sc. I enjoyed the mentorship of Dr. Per Aspenberg, professor in orthopedics who always made his lab members a priority, and Dr. Gunnar Kratz, professor in plastic surgery who before it became fashionable believed that somatic cells had a great, untapped plasticity. He also combined science with fun, and I doubt that I will have the footage of any other advisor putting me on an operating table with marks for incisions for breast implants, and a silicon implant resting on my abdomen.

Even before the day I arrived here at Hopkins, the great administrative staff that we have at the Department of Materials Science and Engineering have helped me with all the paperwork associated with coming in as an international student, made sure that I'm registered for the courses that I am required to, helped set everything up regarding my fellowship funds, and made sure that I meet all the deadlines for my dissertation. A special thanks to Jeanine Majewski, Marguerite Weaver, Ada Simari, Brooke Mesta and Amanda Gursky.

Here at Hopkins, I have enjoyed a strong collaborative environment that I have taken full advantage of. Whenever I have a problem, there is always a lab I can walk to and get expert advice. Bipasha Mukherjee Clavin in Dr. Lee's lab has patiently provided me with stem cell-derived Schwann cells, Matthew Dallas and Xuesong Jiang taught me flow cytometry, Jesse Placone helped me with fluorometry, Brian Chaikind and Nirav Shelat were always there for my cloning questions, Ying Li taught me how to isolate oligodendrocyte progenitors, Kristen Kozielski and Stephany Tzeng in Dr. Green's lab helped me with figuring out nanoparticle carrier combinations, Valentin Sluch taught me

how to make lentiviruses, Erin Pryce and Dr. Michael McCaffery were always helpful with imaging, as was Carol Cooke, and Antje Arnold and Dr. Piotr Walczak helped me with engraftments into shiverer mouse brains, Jean-Philippe Richard provided me with human motor neurons, and Sarah Gross and Akshata Almad helped me figure out so many stains and antibodies to use. Dr. Daniel Hoeppner helped me with technical advice for some ideas I had and I have had many conversations with him, always rewarding. I would also like to thank Constantin d'Ydewalle and Jeremy Sullivan who have always been there to answer my questions regarding molecular biology assays and techniques.

I have also had the fortune to work in two great labs, with great co-workers. I would like to thank everyone in the Mao lab in Materials Science and Engineering, with a special thanks to Russell Martin who helped me find my first apartment and has continued to always be helpful in experimental work, John-Michael Williford who has always provided great insights and comments, Yong Ren has helped me with questions regarding chemistry, Rishab Shyam for many great conversations on the shuttle after long days, Yong-Juan Ren for getting me started on the Schwann cell work, Xiaowei Li and Jisuk Choi for help with hydrogel compositions, José Roman and Charles Hu for making sure that I get out of lab every once in a while, and everyone else for making this a great place to be.

I would also like to thank my other lab, the Höke lab in Neuroscience. A special thanks to Ruifa Mi, without whom only half of this work would have been accomplished. He taught me isolation of both DRG neurons and Schwann cells, and performed all survival surgeries that we conducted. I would also like to thank Nicole Reed for teaching

me the basics of immunochemistry and tissue processing, and Chris Cashman and Jami Scheib for many great and thought-provoking conversations.

I have been very fortunate in my fellowship applications. These have first and foremost secured me a place here at Hopkins, but also given me the chance to design the projects with great freedom. I have received very generous contributions from Stiftelsen Olle Engkvist Byggmästare, the foundation Blanceflor Boncompagni-Ludovisi neé Bildt, and Hans Werthén Fonden.

Finally, I would like to thank my two inspiring mentors. Dr. Ahmet Höke has been my co-mentor, always welcomed and encouraged my questions, and taken me into his lab with the same warmth that he shows to the others in his group. Dr. Hai-Quan Mao was the reason I came to Hopkins, as I had heard that he was a very student-oriented professor, something he has surely lived up to. He has always provided constructive criticism to my academic pursuits, opened up paths for me to run both the project and to prepare for my career, and provided the project with a much-needed positive outlook. When he was presented for a talk a few years back, it was said that he is one of the nicest guys you will ever meet, a statement that I can only echo.

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CHAPTER 1 - PERIPHERAL NERVE REGENERATION AND CELL THERAPIES

This dissertation is motivated by two facts regarding peripheral nerve regeneration. The first is that it exists; the peripheral nervous system (PNS) has an intrinsic capability to regenerate. This is manifested for instance by surgical techniques that now allow for hand, forearm, and face transplantations with accompanied functional recovery, of which over 100 cases have been reported to date[1]. This sets the PNS apart from the central nervous system (CNS), which lacks many of the features enabling regeneration in the PNS, with an injury response that impedes axonal regrowth through the injured and scarred tissue. The second fact that is the basis of this dissertation is that the regeneration is typically incomplete, lacks the necessary specificity and functional recovery, and is highly dependent on the type of injury, nerves affected, distance to the target tissue, and the timing of the surgical intervention[2].

In this chapter, we will first discuss general mechanisms of PNS regeneration, as they are understood today, and some of the main challenges that hinder complete functional regeneration. We will highlight the role of the Schwann cells (SCs), the cell type in focus of this dissertation, in the context of its PNS regeneration. We will contrast the pro-regenerative response of the PNS to the injury response of the CNS to highlight some key aspects necessary for nerve regeneration to occur. Understanding these aspects may allow extending results related to the approach presented in Chapter 4 into regenerative therapies for the PNS and the CNS both. We will then go into greater detail regarding SC development, which is important to understand, as we will be dealing with

stem cell-based approaches for generating these cells. Cell reprogramming techniques will be reviewed in order to understand the capabilities and challenges of methods underlying the cell preparations that we are working with. Finally, the state-of-the-art regarding stem cell-based approaches in PNS regeneration will be reviewed, highlighting the need for and difficulty in deriving fully functional and mature SCs. As these concepts are interconnected, please excuse some overlap between the sections.

1.1 Peripheral nerve regeneration mechanisms

Injuries to nerves in the PNS will lead to the activation and recruitment of a number of cell types. First, the affected neurons will switch from their normal signaling phenotype to a regeneration phenotype with the upregulation of multiple regeneration associated genes[3]. They form endbulbs at the end of their severed axons, at which local peptide translation and secretion takes place[4]. An example of this is the local expression of calcitonin gene-related peptide (CGRP), which may act as a vasodilator[5,6], but also becomes a SC mitogen[7].

SCs become activated and transdifferentiate into a repair phenotype[8-10] (reviewed by Jessen and Mirsky[11]). Myelinating SCs associated with the severed axons will initiate myelin clearance through autophagocytosis of their own myelin components[12,13]. The activation is not only seen in myelinating SCs of injured axons, but also in non-myelinating SCs of nearby tissue[11,14,15]. The activated SCs will play a large part in the repair process through secretion of trophic factors and cytokines. These will both promote the survival of neurons, guide their regrowth, and recruit macrophages to clear debris[16-21]. Recruited macrophages will react to the hypoxic environment at the injury

site and secrete VEGF-A to attract endothelial cells. These endothelial cells together with fibroblasts in turn guide the activated SCs, along which regenerating axons follow[22-24]. Where basal lamina still remains intact from previous axonal tracts in the distal nerve, SCs will obtain a bipolar, elongated morphology that helps guide axons. These structures are called bands of Büngner[25,26]. SCs produce extracellular matrix components such as laminin, which has been shown to help axons regenerate[3], and provide modality-specific tropic cues for sensory and motor axons, with a strong upregulation of neurotrophic factors in the distal nerve[16,17,27].

An important point to keep in mind is that in the PNS, the axons extend over vast distances, with the perikarya residing in or near the spinal cord. Therefore only the axon proximal from the site of injury will remain connected to the neuronal cell body. The distal part will undergo a process termed Wallerian degeneration in the case of transections injuries, or Wallerian-like degeneration in crush injuries[25,26,28], under which the distal axons are cleared through an internal axonal breakdown followed by recruitment of immune cells[29]. The breakdown of distal axons, infiltration of myeloid cells, and clearance of debris from the distal nerve is necessary for regeneration to proceed[30-34].

1.2 Challenges facing peripheral nerve repair and functional regeneration

The rate of nerve regeneration has been found to be between 1-4 mm per day, correlating with the rate of slow axonal transport of important cytoskeletal proteins synthesized in the perikarya[2,35]. The limited extension rate becomes a problem as the

chronically denervated distal nerve has a declining ability to support regeneration[36]. While there is a robust upregulation of trophic and tropic factors in the distal stump at first, this is not retained over long periods of time and the axons lose the attractive cues that promote their growth[36,37]. Furthermore, while there is a strong angiogenic response in the acute phase[5], chronically denervated nerve trunks become ischemic with a declining perfusion through the vasa nervorum[37]. Guidance cues for axons are also lost through SC atrophy with an associated loss of Büngner bands and basal lamina tracts[38-40].

Chronically denervated muscle tissue also undergoes atrophy, and the number of spared muscle fibers is correlated with the number of innervating axons, showing a required nerve input for muscle tissue maintenance[41]. The capability of motor endplates to be reinnervated also declines over time of denervation[42]. Muscle atrophy has been reduced by engrafted embryonic motor neurons, or motor neurons derived from human pluripotent stem cells[43-46]. A recent study showed that direct depolarization of muscle fibers using optogenetics reduced the muscle atrophy[47], suggesting that the atrophy results at least in part from lack of depolarization and muscle contraction. Ectopic innervation and long-term stimulation however seems to reduce receptivity to reinnervation in chronically denervated muscle[48], suggesting that other interventions are needed to maintain motor endplates receptive of reinnervation, or to provide the regenerating axons with appropriate signals to efficiently innervate. The limitations on axonal regrowth rate and declining support thereof coupled with end-organ atrophy make regeneration over long distances extremely challenging in the PNS, and a reasons why injuries more proximal to the CNS generally display poorer functional outcomes compared to more distal ones.

Another challenge lies in the specificity of the regenerating axons, both in terms of discriminating between sensory and motor pathways, and to avoid innervating antagonizing muscles with the same motor neuron pool. The control over motor vs. sensory specificity in reinnervation is thought to be controlled through the SCs. In a process named preferential motor reinnervation (PMR), Brushart *et* al. observed that motor axons preferentially regenerate through motor tracts in a femoral nerve transection model, even if the sensory and motor branches were purposely misaligned[49]. The process of correct motor nerve target innervation was improved over time, with collateral projections form the same motor neuron extending into both the motor and sensory branches having the incorrect collaterals removed, suggesting a pruning of incorrect innervation into sensory trunks[50]. As PMR takes place even when access to the target end-organ is removed, the process appears inherent to the nerve trunk modality[51]. Furthermore, SCs have been shown to differ in their phenotype and injury response depending on their associated axons, secreting modality-specific tropic factors[17,52,53]. After spinal root avulsion, where no or very limited axonal contact is retained with supporting SCs, motor nerve grafts have been shown superior to sensory nerve grafts in promoting motor neuron survival[54]. The modality specificity is thus not only important for path finding, but also for support of neuron survival. These effects may be even more evident in the spinal root avulsion model where the injury is very proximal to the perikarya and there is very limited modality-specific SC support of the neurons retained. The ability of nerve trunks to support PMR has been found to decline with age[55].

In terms of target muscle innervation, a comparison between nerve transfers to direct repair is useful to understand the problems faced in repairing nerves with multiple

antagonistic target muscles. Intercostal nerve transfer is a procedure that may be employed in severe injuries to the brachial plexus, nerves innervating the hand and arm. Here the intercostal nerves, which control chest expansion for breathing but can be spared, are surgically transferred to innervate the target muscles of the brachial plexus. In doing so, entire motor neuron pools are transferred to innervate and serve the same target muscle, and the brain can adapt to controlling the movement of its new pathway through central compensation. The key here is that the entire motor neuron pool, *i.e.* collection of lower motor neurons receiving the same input signal, will serve one target muscle. In the case of nerve repair however, motor units (individual motor neurons that innervate a group of muscle fibers and constitute subdivisions of the motor neuron pool) from different motor neuron pools may get mixed, resulting in the same input signal contracting antagonizing muscles and reducing or abolishing the movement function. This is an especially challenging aspect of nerve repair, as there does not seem to be any plasticity at this level able to rescue or re-learn mixed projections from a pool of lower motor neurons[56].

As in most tissue, the regenerative capacity of the PNS diminishes with advanced age. This is due to a multifaceted reduction in the repair response, but current evidence suggests that factors in the end organ, distal nerve and immune response fall short rather than factors intrinsic to the regenerating neurons. At least these factors dominate over those intrinsic to the neurons. The extension velocities of young and old axons are the same, but axons growing through an old nerve appear to follow a less direct path, navigating around debris[57]. The gene expression profiles of neurons before and after injury have also been found to be similar between young and old mice[58]. On the other

hand, grafts from young into old animals lead to a more robust response both in terms of innervation, debris clearance[58-60], and support of PMR[55], arguing that mechanisms intrinsic to the young graft are at play in the enhanced regeneration. What is clear is that SCs less efficiently undertake a repair phenotype with advancing age. A key observation supporting this is the delay with which *cJun*, a transcription factor orchestrating the transdifferentiation into the repair SC phenotype[8], becomes uppregulated[58]. All other observations in terms of impeded debris clearance, upregulation of injury response markers, and reduced macrophage infiltration may be resulting from this, although no causal link has been directly shown and effects from other cells involved are not to be excluded. The difference in the role of macrophages is an unresolved issue. It is clear that debris clearance is less efficient in aged animals[58-60]. This could be due to both a reduced recruitment and a reduced functionality of the macrophages in aged animals. The former could further be due to inefficient signaling either from cells recruiting them to the site, such as SCs, or intrinsic to the macrophages to respond to the signaling and migrate to the site. The lab of Ahmet Höke reported a reduced *in vitro* phagocytosis by macrophages isolated from aged rats as compared to young animals[59], pointing to a mechanism intrinsic to the macrophages' functionality. In the study by Painter *et al*., they could not rescue the impaired clearance seen in aged mice through heterochronic parabiosis with a young animal, from which they excluded macrophage-intrinsic mechanisms[58]. However, the graft will still contain old cells that may be more inefficient at the recruitment of the young macrophages to the site. By pharmacological blocking of macrophage activation it has been shown that a reduced activation and recruitment clearly affects the myelin clearance also in young animals[60]. While a reduced recruitment

in old animals could not be resolve in the study by Scheib *et al*., there was a reduced RNA expression of several cytokines responsible for macrophage recruitment[59]. Inefficient macrophage recruitment could also extend to delayed revascularization of the graft[22]. In conclusion, current evidence suggests that age-dependent decline in PNS regeneration is strongly associated with a declining capacity of the distal nerve to support regeneration, but that the neurons maintain a robust repair response. It is however unresolved if the entire impairment is manifested in the distal nerve tissue, or if there is an immune cell-intrinsic component.

Injuries of large portions of nerves become challenging to manage surgically. Up to a certain size, the damaged tissue can be removed and direct nerve anastomosis performed between the nerve stumps. If large portions of nerve tissue have to be removed, the ends cannot be brought together directly due to the tension in the tissue from doing so will contract blood vessels and restrict blood flow to the tissue. In this case, a nerve graft or nerve transfer has to be employed[61]. The current gold standard for very large defects is to use an autografts, where nerve tissue is taken from less critical places in the patient and engrafted to the site. In doing so, the tissue already contains the nerve architecture with basal lamina tracts as well as support cells such as SCs, fibroblasts, endothelial cells and macrophages, which creates a graft vastly superior to any acellular graft. There is however a limit to how much suitable tissue that can be spared, and there are also complications with morbidity at the donor site. Much effort has thus been put forth to create good alternatives by using synthetic grafts or decellularized tissue[62]. While acellular grafts over short distances can be repopulated by SCs migrating from the host tissue at the ends, studies in mice suggest that cellular

senescence eventually restricts the ability of host cells to repopulate the graft[63]. It should be noted that it remains unclear if this mechanism translates to rat or human SCs. *In vitro*, SCs from rats and humans have better expansion capabilities than those derived from mice[64-66], but any direct comparison has not been made in terms of *in vivo* senescence. The performance of synthetic nerve guides can therefor be greatly enhanced by pre-seeding them with support cells, ideally SCs due to their importance in orchestrating the regenerative response and remyelinate axons. The availability of autologous SCs is restricted for the same reasons as for autografts. This has created a need to identify alternative sources of SCs, such as through cellular programming from other cell types[1,62,67].

1.3 Contrast to the central nervous system

The response to injuries is different between the PNS and CNS, as are the prospects for functional regeneration to take place. This is not due to an inability of the CNS neurons to regenerate, but rather an effect of the tissue, immune cell and glial response to injury in the tissue. Two seminal studies demonstrated this by showing that CNS neurons could extend robustly through PNS grafts in settings where axonal regeneration would normally not take place in the CNS[68,69]. Engraftment of adult DRG neurons into the corpus callosum later demonstrated that when this was done successfully in a minimally invasive manner that did not elicit an injury response and upregulation of growth-inhibitory chondroitin sulfate proteoglycans (CSPGs), graft axons could extend along white matter tracts of the brain. When the engraftment elicited a reactive response, the axons were halted at the graft/host interface[70]. These studies demonstrate that the tissue response to injury differs significantly, in a way that for PNS tissue permits

regeneration while in the CNS a barrier is formed through which axons cannot extend under normal conditions. This barrier is known as the glial scar, which serves to re-establish the compromised blood-brain barrier. The scarring has been shown necessary, as vast inhibition of scar formation increases the extent of tissue damage[71-74]. It is comprised of a multitude of cell types that upregulate and deposit multiple extracellular matrix components inhibitory of axon extension. We will not discuss the details of the scar formation in this dissertation, but there are some excellent recent reviews of the topic to be found[75-78]. Instead we will focus on a couple marked differences in the injury response that may explain why CNS axons that fail to regenerate through injured CNS tissue may be able to extend through PNS grafts.

First, there is a great difference in the response of the respective myelinating glia. While SCs associated with injured distal axons will actively clear the myelin, first by an autophagocytotic process and then by active myelin debris clearance and immune cell recruitment[8-10,12,13], myelinating oligodendrocytes seem to serve no active role and frequently undergo apoptosis upon denervation after spinal cord injury[79-81]. In the CNS, oligodendrocyte progenitor cells (OPCs) become activated to migrate towards the lesion site and produce both new OPCs as well as oligodendrocytes[76,82-84]. Dystrophic axons have been found associated with $NG2^+$ and $vimentin^+$ cells at the penumbra of the lesion, the edge that is otherwise non-conductive of axonal growth. Vimentin is expressed by multiple cell types, including OPCs, macrophages, ependymal cells and pericytes[83], and NG2 is a cell-surface CSPG found on macrophages and OPCs[85]. Thus, at least a subset of these cells seems to stabilize the dystrophic axons. It remains unresolved which lineage these cells come from, but it has been proposed that axons may synapse onto

$NG2^+$ cells, both stabilizing them but also preventing them from later axonal extension[76,83].

There is also a marked difference in how the CNS and PNS facilitate Wallerian degeneration, which is significantly slower in the CNS. Upon PNS injury, the blood-nerve barrier opens up distal to the site of injury to allow for efficient infiltration of immune cells and clearance of myelin debris in the distal nerve[28,86]. The blood-brain barrier of the CNS however remains intact distal from the injury, maintaining the integrity necessary for neuronal survival but also limiting the ability to clear myelin associated with dystrophic axons by invading macrophages[76]. It also prevents the opsonization of myelin debris by complement factors and antibodies from the blood, which has been shown as a key mediator of myelin phagocytosis by macrophages[34,87-91].

The PNS and CNS microenvironments also have a marked impact on the phenotype of macrophages and microglia. Macrophages have been classified according to their activation state, from inactive M0 to the two activated phenotypes M1 and M2. Without going into detail regarding these phenotypes, the activation is mediated by distinct cytokines, where M1 is seen for instance upon IFN-γ exposure, rendering the cells to secrete pro-inflammatory cytokines, cytotoxic mediators such as reactive oxygen and nitrogen species, and increasing their phagocytic and antigen-presenting capacity. M2 polarization results from for instance IL-4 exposure, upon which M2 macrophages reduce their production of inflammatory cytokines. In non-neural tissue the M2 macrophages have been found important for dealing with extracellular pathogens[77]. Macrophage polarization is therefore controlled by the microenvironment in which they act, and studies pre-incubating macrophages with PNS tissue prior to engraftment into

spinal cord[92] or optic nerve[93] injuries led to enhanced regenerative outcomes. However, as neither study used a non-stimulated control or macrophages stimulated by CNS tissue, but rather did non-cellular mock injections, a direct causal link by the stimulation could not be established. It can be argued that improperly polarized macrophages will be detrimental to CNS cell survival, as secreted factors such as reactive oxygen and nitrogen species will cause secondary damage[76,77]. Engrafted M2 polarized macrophages also mostly fail to retain their polarization over long periods in the CNS, arguing that the tissue promotes an M1 or non-polarized phenotype over an M2 phenotype[94]. The site of a spinal cord injury is essentially deplete of IL4, delivery of which causes macrophages to undertake an M2 phenotype, with reduced tissue damage and enhanced recovery as a result[95]. To summarize, the Wallerian degeneration is much more effective in the PNS than in the CNS, largely due to the response of the glial cells, where SCs of the PNS take upon an active role. There is also an important contribution from tissue access from blood-derived macrophages and opsonins, as well as the local environment's effect on the polarization contributing to a more rapid clearance of debris in the PNS. While the myelin debris is cleared from the distal nerve of the PNS in 1-2 weeks[96,97], myelin debris may persist for years after CNS axon degeneration in humans[98-100].

There are also structural differences between the CNS and the PNS that impact axonal regeneration and directional regeneration. SCs form tracts of basal lamina, with a laminin-rich tube surrounding myelinating SCs. After Wallerian degeneration, SCs will align within these tracts in bands of Büngner[25,26]. As oligodendrocytes lack such basal lamina structures, they also lack these structural cues of directional guidance that also isolates the regenerating axons from exposure to inhibitory molecules. The bands of

Büngner may contain cytokines assisting regeneration[2], and laminin itself has been shown beneficial to axonal regeneration in the PNS[3,101].

The differences between CNS and PNS tissue regeneration is useful for finding interventions both for CNS and PNS regenerative therapies. The obvious approach is to study the regenerative potential of the PNS, figure out what factors that are at play, and if these aspects can be used to promote CNS regeneration. With this in mind, the classical engraftment models with PNS tissue interfacing the brain[68,69] should be re-visited in light of current techniques and understanding of the microenvironment's impact on immune cells and glia, looking more in-depth at how the PNS tissue may affect the border region from which neurons extend their axons into the graft. A less obvious lesson is how aspects learned from CNS injury and disease can be used to promote PNS regeneration. In Chapter 4 of this dissertation, we demonstrate that a transdifferentiation of OPCs into SC-like cells seen in certain experimental demyelination models[102-104] and in patients suffering from multiple sclerosis[105,106] also could occur upon heterotopic engraftment of OPCs into the PNS, resulting in remyelination of regenerating axons by SC-like cells derived from the graft. This type of fate specification due to environmental factors can also be leveraged for CNS applications in the classical way of providing more pro-regenerative phenotypes in support cells. Recent reports on axonal extension through glial scar where cytokine-laden hydrogels were employed in the model failed to consider the contribution these had on resident and recruited glia[107,108]. The key to these findings may not be intrinsic to the regenerating neurons, but to the recruitment, phenotype and polarizations adapted by glia and macrophages respectively to support the regeneration.

1.4 Schwann cell development

SCs develop from the neural crest stem cells (NCSCs), which is a transient cell population forming at and migrating out from the border between the neural plate and the newly fused neural tube in its most dorsal region. They will give rise to a vast array of cell types, including melanocytes, cardiac cells, neurons and glia of the PNS. The origin and migratory path of the cells will play an important role in their fate commitment, with the NCSCs that give rise to SCs arising from the trunk cells[109]. The specification of cells into NCSCs is induced by a combination of Wnt, BMP and FGF signaling that activates an intricate gene regulatory network[110]. FGF signaling induces the expression of *Hairy2* (a *Hes1* ortholog in Xenopus) to attenuate BMP signaling in the initial neural plate border gene upregulation[111,112]. Wnt signaling then allows *Pax3* and *Zic1* to induce the expression of a series of neural crest specifiers[113-117], while BMP signaling induces *Id3* expression to inhibit the activity of *Hairy2* and allow neural crest specification to proceed[111]. The sequence of transcriptional regulation events initiated will eventually result in the upregulation of the core transcription factors *Sox10* and *FoxD3*, the expression of which are maintained throughout the specification of SCs[118,119], and marks the specification of NCSCs from the neural plate border.

The newly specified NCSCs will now undergo an epithelial-to-mesenchymal transition to migrate away from the neural tube, followed by specification into their different cell types. For the purpose of this dissertation, focus will be on the specification into SC precursors. Following the specification of sensory neurons which reside in the developing dorsal root ganglia (DRGs), the cells that will be specified into SC precursors migrate through these structures and interact with peripheral axons[110]. It still remains

unclear what triggers this specification, as the transcription factors known to regulate this process (*Sox10, FoxD3, AP2α* and *Pax3*) are also expressed in other NCSCs that do not give rise to SC precursors[120]. *Sox10* has been found to play a key role. It upregulates the expression of the neuregulin receptor *Erbb3*, which controls not only specification but also makes the specifying SC precursors receptive to axonal support necessary for their survival[121-128]. The level of *Sox10* is controlled by a mechanism involving histone deacetylases HDAC1/2, where the *Pax3* promoter is activated through interactions between *Sox10* and HDAC1/2. *Pax3* then synergizes with *Sox10* for enhanced levels of *Sox10* expression associated with glial specification[129]. While BMP signaling is inductive of neuronal fate[130-133], this is suppressed by FGF2 and activation of notch signaling for glial fate commitment[134,135]. Furthermore, maintained expression of the transcription factor *FoxD3* is necessary for glial fate commitment over neuronal or melanocyte specification[119,136].

While the SC precursors are multipotent, giving rise to both mesenchymal stem cells, fibroblasts and parasympathetic neurons in addition to SCs, the next step in development into immature SCs is through to be irreversible. The cells also switch from an axon dependence for their survival, to autocrine secretion of survival factors[137]. The timing of *AP2α* downregulation in PNS development, along with *in vitro* results showing reduced differentiation of SC precursors into immature SCs when its expression is enforced, suggests that it is a negative regulator of the transition into immature SCs[138]. Notch signaling in SC precursors leads to downregulation of *AP2α* and an enhanced expression of the neuregulin receptor *Erbb2*, making the cells more responsive to neuregulin signaling and promotes proliferation of the cells[139]. Once committed, the

immature SCs cease to migrate and associate with axons to undergo the process of radial sorting, where SCs take on a one-to-one relationship with axons to be myelinated, or embrace multiple small caliber axons into non-myelinated Remak bundles[109].

1.5 Cell reprogramming and engineering for regenerative medicine

As already exemplified for PNS tissue in the discussion above, tissue regeneration does not take place for large injuries in most types of human tissue, liver being one exception. To overcome the limited regenerative capacity, stem cell- and reprogramming-based approaches in tissue engineering and regenerative medicine rely on deriving human cells that can provide supportive cues to endogenous cells, and/or replace lost cell types, in order to regenerate tissue. To achieve this, we need to produce the specified cell type from cells that can be obtained in a minimally invasive manner, and expand them to sufficient quantities. Unless expandable progenitors can be isolated, this typically relies on reverting cells into a progenitor state, or propagating a different cell type that then can efficiently be converted directly into the target cell type.

The cellular plasticity that enables these, often drastic, conversions of cellular identity has not been obvious. Through studies of amphibian embryogenesis, it was believed that already in the late gastrula the cells began to be irreversibly committed to specific fates, and information required to generate other lineages was lost from the nuclei as the cells differentiated. An at the time new technique called somatic cell nuclear transfer had enabled introduction of a cellular nucleus into an enucleated egg[140]. As nuclei from blastocysts could generate normal, healthy tadpoles, while those from the late gastrula were not able to do so more than infrequently, and it was never successful

beyond stages post-neurulae or later in the studies by Briggs and King, it was concluded that nuclei became differentiated and lost the information necessary to generate cell types beyond their specification[141]. These views however became challenged by the results of a graduate student named John Gurdon in Michail Fischberg's lab. While the studies by Briggs and King were conducted in the amphibian *Rana pipiens*, Fischberg's lab had switched to using the African frog *Xenopus* as they found benefits with their short time to reach sexual maturity, laboratory housing requirements, and ability to produce eggs all year around as opposed to the seasonal reproduction of *Rana pipiens*. It turned out that, rather serendipitously, *Xenopus* was also better suited for successful somatic cell nuclear transfer from later-stage cell types[142]. While most tadpoles were still abnormal, the finding that nuclei isolated from pre-hatching tadpoles[143] or tadpole intestinal cells[144] could occasionally result in normal tadpoles and frogs supported the conclusion that even differentiated cells retain the information necessary to produce cells of all lineages of the adult animal; that the nucleus had not irreversibly lost any information, but that it was merely using select parts of it specific for the target cell. This finding is the basis for all cellular reprogramming efforts thereafter, where manipulations can alter the gene expression profiles of cells to change their identity.

A number of methods to make cells change their identity have since been explored. Access to human pluripotent stem cells, the progenitor of all cell types in the adult human body, was gained following their successful isolation from the inner cell mass of blastocysts[145]. These cells could be cultured in clusters called embryoid bodies that mimic their organization in embryonic development. Through addition of specific growth factors, the cell fate can be directed by recapitulating the cues found *in vivo*. Such

techniques have been employed for instance in the generation of different neuronal cells from human pluripotent stem cells[146-148].

Another commonly utilized approach is to enforce the expression of the downstream transcription factors activated by these signaling pathways to direct cell specification. The first demonstration of this however relied on two other seminal findings. The first was that treatment of immortalized fibroblasts with 5-azacytidine, shown to incorporate into the DNA, resulted in transdifferentiation into chondrocytes, adipocytes and myocytes. It was noted that time was required for the cells to undergo division[149], and we today know that the incorporation of 5-azacytidine into DNA blocks DNA methylation by methyltransferases that act upon cell division, thereby changing the genes available for expression[150]. The other finding was that mature somatic cells also contained trans-acting factors that could induce gene expression of their lineage in the nuclei from other cell types. This was done through fusion of two cells, in this case human amniocytes to mouse muscle cells, in a way that kept their respective nuclei separate and intact, upon which the human nucleus expressed genes related to the muscle cell[151]. It was then possible to isolate the transcription factor responsible for inducing muscle cell gene expression in 5-azacytidine-treated fibroblasts. Screening fibroblasts for myosin heavy chain expression after transfection with a myocyte cDNA library identified the factor *MyoD*, which was capable of converting immortalized fibroblasts into myoblasts similarly to what had previously been observed after 5-azacytidine treatment of the same type of cells[152]. Many years later in 2006, a study identified a combination of four transcription factors that could convert mouse fibroblasts into so called induced pluripotent stem cells (iPS cells) by screening for combinations of 24

transcription factors known to be responsible for embryonic stem cell maintenance using lentiviruses to overexpress these constructs[153]. The following year the same had been accomplished for human cells[154-156], thus constituting a source of cells that can be produced in a minimally invasive manner, and produce all the cell types of the adult (even though this has not been strictly shown for human cells due to obvious restrictions to doing so *in vivo*). Transcription factor overexpression is therefore a tool by which core gene regulatory networks can be induced using a part of the cells' intrinsic gene regulatory machinery, bypassing extrinsic signaling cues that would normally determine its specification, and forcing the expression of fate-determining factors which are not present in the starting cell type. This has been employed to directly convert fibroblasts into neurons[157,158], and B cells into macrophages[159,160], to name a few examples.

Reprogramming techniques are now emerging as clinical therapies. After encouraging results of maintained vision in rat models of age-related macular degeneration (AMD)[161], retinal pigment epithelial (RPE) cells derived from human embryonic stem cells have shown promise in phase I and phase II clinical trials for the treatment of dry AMD and Stargardt's macular dystrophy[162,163]. While injection of cells in suspension gave integration into the host macula, the coverage was patchy[163]. Efforts are now underway to develop cell culture scaffolds that allow for proper organization of the derived cells in culture, and to improve the coverage by providing a support matrix to facilitate the engraftment[164,165]. Efforts like these to create better *in vitro* culture systems will be crucial to improve the quality of the cell preparations. An example of where the quality and purity of the engrafted cells have shown to be important is for the treatment of Parkinson's disease (PD). In classical PD, A9 dopaminergic neurons of the *substantia*

nigra pars compacta are lost, interrupting the dopaminergic input from the nigrostriatal pathway to the dorsolateral striatum that regulates motor function[166]. Clinical trials engrafting human fetal midbrain tissue provided evidence for the use of cell therapy to treat PD, but the results were varying between patients, with some showing significant improvement while others had no improvement and "troublesome dyskinesia in a significant proportion of patients after transplantation" was observed[167]. This was likely due to the heterogeneous graft material, where the mDA neurons make up only a fraction of the cell population[166,168].

All but one of the clinical trials involving cells derived from pluripotent stem cells have used embryonic stem cells as their starting material, with only one patient in Japan receiving iPS cell-derived RPEs[169]. While the first methods for deriving iPS cells relied on viral vectors for the transcription factor overexpression, non-viral[170-172] and non-integrating[173] techniques have since been developed to overcome the risk of insertional mutagenesis and oncogene activation. Nevertheless, there are concerns regarding the genomic stability of iPS cells. The maintenance of site-specific methylation has been estimated to have fidelity of 90%-98%. Thus, vast expansion of progenitor cells can and will lead to random and unpredictable changes in the DNA methylation which may affect their gene expression[174-176], and clones with favorable gene expression patterns or chromosomal rearrangements get a growth advantage. This can for instance be through a common partial gain of chromosome 12, which has been shown to increase their expression of *Nanog* and *Gdf3*[177,178]. The reprogrammed cells may also have erased their epigenetic marks more or less efficiently, leading to clones that carry a memory of their starting cell type[179-181]. This leads to differences in the quality of iPS clones, and some

more or less biased to differentiate into certain lineages. In order to robustly and reproducibly derive cells with a predictable functionality from different individuals, these challenges and shortcomings in reprogramming need to be overcome. Extended culture has been shown to ameliorate some of the somatic cell memory seen in iPS clones, potentially due to incomplete reprogramming at early passages and continued selection for clones under culture conditions supporting pluripotency[182-185], but it is also associated with the risk of accumulating mutations that may be detrimental or even dangerous. Some of the lineage bias between lines may be difficult to completely overcome, as data suggests that iPS cell heterogeneity is largely due to disparities between donors and not tissue of origin[186,187]. A potential approach is to use *in silico* analysis of both gene expression, global DNA methylation and histone modification profiles to predict the differentiation potential of iPS clones[185,188]. For clinical applications, both the effects from aberrant epigenetic profiles and mutations associated with the reprogramming process and cell expansion, as well as stable cell fate commitment need to be addressed to avoid transplantation of tumorigenic cells to the patients[189,190].

Finally, a major limitation with current cell programming and derivation methods is that they tend to produce immature progeny, rarely reaching cell phenotypes and mature functions seen beyond the perinatal stage[191]. This is especially challenging for organ systems that continue to mature and develop well past birth, such as the nervous system. Recent advances in organoid technology have however created methods to study prenatal development of a range of tissues through liver[192], pancreas[193,194], stomach[195], and brain[196] organoids to name a few examples. This way, composite tissue from early development can be created in the lab, composed of the relevant cell types for that organ

system[197,198]. Nutrient diffusion and waste transport however restricts their *in vitro* culture, as the construct is not perfused and eventually forms a necrotic core. This limits the size and duration over which the organoid development may proceed[198]. Even if endothelial cells were to form a vascularized construct, perfusion would not happen, as there is no heart. This has created a biomedical engineering challenge to integrate the organoid with a system to perfuse it without perturbing the culture requirements allowing for the cells to self-organize, or to subdivide established organoids into sizes that are no longer diffusion-limited while retaining essential tissue organization. There are occasions where composite cellular grafts will be beneficial, as exemplified by engrafted liver organoids that successfully integrated with the host vasculature[192]. However as described above, fetal tissue-derived grafts have been limited in their applicability for the treatment of PD, likely due to their heterogeneity[166-168]. In these cases organoids may serve as culture vehicles from which the desired cell population can later be isolated. Heterogeneity is not a problem only inherent to organoids. Many derivation protocols also end up with a sizeable population of non-specific progeny, requiring purification steps to isolate the target cells[199].

In conclusion, cell programming techniques are widening our abilities to provide cell therapies for regenerative medicine where suitable cell sources cannot be directly isolated. Some major challenges that remain include the robustness and specificity of the programming methods, and to derive functionally mature progeny past embryonic development.

1.6 Stem cells for peripheral nerve repair

A vast number of different stem cell sources have been employed in experimental models of peripheral nerve repair. They all however follow a hierarchy of support mechanisms, which is 1) extracellular matrix deposition and remodeling, 2) secretion of cytokines, exosomes, and neurotrophic factors that influence both regenerating axons and support cells, and finally 3) cells that can remyelinate the regenerating axons. While pretty much any cell that can survive engraftment can be argued to act through the first two mechanisms to some extent, only very few cell types and sources have been shown capable of remyelinating the regenerating axons.

While neural stem cells (NSCs) cannot be isolated in a non-invasive manner, they can be readily induced from pluripotent stem cells[200-202], thus comprising a potential cell source for peripheral nerve repair. A study conducted using a neural stem cell line found them capable of promoting regeneration in a rat chronic denervation model[203]. 4 month after engraftment, only about 0.5-1% of the engrafted NSCs were found to remain in the tissue, and they all were found positive for the neural stem cell marker nestin. The main supportive effects must therefore have taken place at earlier phases. *In vitro* analysis showed the cells to express MMP-2, suggesting that they can remodel the extracellular matrix. Transfection of the cells to overexpress GDNF didn't show any significant additional improvement. In light of previous reports of these cells expressing large quantities of trophic factors[204], the naïve cells may therefore have already had a robust trophic support rendering the GDNF transfection redundant. The study also demonstrates that cell therapies may be an effective way to provide the trophic support lost in the distal nerve over prolonged denervation[36,37].

Mesenchymal stem cells (MSCs) comprise a very commonly employed cell source. They can be isolated from for instance blood, Wharton's jelly of the umbilical cord, adipose tissue and bone marrow. While cells from these different sources also differ in their differentiation potential and phenotypes, we group them together in the scope of this discussion[1,205]. The cells are popular since the naïve cells have consistently been found safe in multiple clinical trials in various applications. Furthermore they have displayed an immune-modulatory effect, reducing the inflammation, although others argue that the cells may rather be immune-privileged, not eliciting an immune response in the host themselves, and whether or not they provide a pro- or anti-inflammatory tissue response depends upon their prior conditioning[1,205]. A common scientific malpractice is therefore to argue for the use of MSCs to act as beneficial immunomodulators without ever confirming that it holds true for the cell preparations used in the studies. To further assume that they are non-immunogenic upon differentiation into other lineages is even less justified. For instance, the immunogenicity of iPS cell-derived cells varies greatly depending on the cell fate they have assumed[206]. The immune tolerance must therefore be evaluated specifically for every application and cell preparation method. For peripheral nerve applications, MSCs are often differentiated into a SC-like phenotype. The first protocol was devised by Dezawa *et al*. in 2001[207], after which over 100 reports employing such cells have been published[205]. Through the differentiation the cells become more potent in promoting nerve regeneration in at least two ways. First, they upregulate multiple neurotrophic factors which greatly improves their ability to support nerve regeneration over undifferentiated MSCs[208]. Second, they have repeatedly been shown to be functional and myelinating[207,209]. Nevertheless, studies comparing the action

of MSC-derived SCs to primary SCs have still found them to be less functional. Axons in nerve guides loaded with MSC-derived SCs do not extend as efficiently as those in nerve guides loaded with primary SCs[210], and ultrastructural analysis of their myelin has shown somewhat aberrant morphologies, especially when analyzed at later timepoints[211]. The phenotypic stability of the cells also depends upon signaling from neurons, as MSC-derived SCs that have been co-cultured with DRG cells do not revert from their SC phenotype to the same extent as cells that have not been co-cultured with DRG cells[212]. These studies have all used murine MSCs in murine hosts. For human MSC-derived SCs, there are so far no reports of functional myelination to the best of our knowledge, although there are studies that have derived SC-like cells from human MSCs[213-215].

Additionally, stem cells isolated from the skin[216], termed skin-derived precursors (SKPs), have been shown to differentiate into SC-like cells[217-219]. Their reports of a functional, myelinating phenotype however requires closer examination. In the report by McKenzie *et al*.[218] they show SKP-derived cells that are associated with axons and also label positive for Ki67, a cell proliferation marker. This is by no means a myelinating cell, as SCs turn post-mitotic upon myelination,[220,221] and perfect co-localization of MBP with the YFP reporter is unlikely. Furthermore, after engrafting the cells distal to the site of a sciatic nerve crush injury in rats, the MBP staining overlapped nearly perfectly with the YFP reporter from the engrafted cells, but there was no MBP signal in nearby nerve tissue[218]. Presumably, the site would have been littered with host SCs also engaging in myelination, so it is highly suspicious that no such signal was seen. A later report by the same lab showed no more convincing evidence[217], and these results simply point to bad staining and imaging practice in the lab rendering these results inconclusive, even though

well-renown journals seem to give them a pass when it comes to overstating their results. They did report convincing evidence of myelination for human SKP-derived cells upon engraftment into the dysmyelinated Shiverer mouse brain. A basal lamina structure could however not be discerned from the images presented, nor any other evidence that the cells retained a peripheral glia phenotype when engrafted into the brain[218]. The phenotypic stability was evaluated by Walsh *et al*.[219]. SKP-derived SCs were engrafted into decellularized sciatic nerve grafts. 10 weeks after engraftment, about 2/3 of the engrafted cells retained an S100β expression, a marker for mature SCs. Their *in vitro* results also demonstrated myelination in a more convincing way than that discussed above.

For human pluripotent stem cells, there are a few reports of SC-like cell derivations[222,223], as well as some reports of direct differentiation from fibroblasts[224,225], but they have shown limited if any functionality in terms of myelination. Two studies indicate possible myelination in co-cultures with rat DRG neurons[223] or human embryonic stem cell-derived NSCs[225], but the neuronal cell preparations cannot be assumed to be 100% pure from myelinating glia, so the lack of reporters in the SCs together with the low observed frequencies of myelination (personal communication with the authors) raise the possibility that the MBP staining was due to contaminating glia.

The overall lack of functionality obtained in human cell preparations can be due to a number of factors. First the effect can be a result of suboptimal *in vitro* culture conditions. The cells could differ in the media requirements from those commonly employed in mouse or rat SC myelination assays. Second, it could be a species mismatch issue, with murine cells or hosts commonly employed both for *in vitro* and *in vivo* studies,

resulting in the potential failure of axon to SC communication hampering myelination. This may be overcome through the use of human cell-derived neurons, but then two additional questions arise: are the neurons of a mature phenotype that can be myelinated, and can co-culture conditions be found where both neurons and glia requirements are met? We tried this with human iPS cell-derived motor neurons in collaboration with the Maragakis lab, but despite several culture conditions tested, none were able to maintain healthy cells over prolonged periods of time. Third, it could be that the stem cell-derived SCs are of an immature, progenitor phenotype, not yet ready for myelination. In the best-case scenario, long periods of contact with axons under the right conditions may allow the cells to mature. Alternatively the maturation could be enforced. Finally, the expression of canonical SC markers is no guarantee that the cells have fully undertaken a SC phenotype. Partial non-SC phenotypes may have been induced, and the cells may be dysregulated in a way that blocks full functionality. Examples of this have been seen before, but it required gene expression profiling and comparison to profiles from multiple tissues to identify an aberrant gene regulatory network node that could be silenced[226,227]. Silencing approaches can however be undertaken in a high-throughput fashion screen of multiple targets without prior knowledge of the underlying gene regulatory network if a robust and efficient functional metric or marker is available. For the reasons discussed above, such an assay is not obviously available for human SCs. The closest would be the dysmyelinated Shiverer mouse brain, where graft-derived myelin could be easily distinguished, but for low frequency events it is cumbersome and the brain microenvironment may have unintended effects on the phenotype of the cells under study.

1.7 Hypothesis and overall objective

The overall objective was to provide a source of functional, myelinating Schwann cells from an accessible source to support regeneration of peripheral nerve injuries. We focused our work on human pluripotent stem cell-derived SC precursor-like cells, under the hypothesis that enabling these cells to reach functional maturation to remyelinate axons also would improve their ability to support peripheral nerve regeneration. We approached this by the method of overexpressing transcription factors that were known to control the maturation and myelination function, targeting cell-intrinsic gene regulatory mechanisms for the cell functionalization. We also aimed to provide tools for achieving transgene overexpression in the target cells using non-viral gene delivery vectors in order to provide more translatable means to promote the cell functionalization.

With a lack of functional, myelinating glia for peripheral nerve cell therapies, we investigated if oligodendrocyte progenitor cells (OPCs), the progenitors of oligodendrocytes that form myelin in the CNS, could survive and be employed in the PNS. With prior reports from focal CNS demyelination models showing this cell type capable of undertaking a SC-like fate[102-104], we further characterized the cell fate commitment in the PNS microenvironment. This is, to the best of our knowledge, the first investigation of OPCs and their functionality in a heterotopic engraftment into PNS tissue.

1.8 Specific aims

Specific Aim 1: Establish an efficient non-viral gene delivery method to achieve controllable transgene expression in stem cell-derived SCs

In the experiments presented in Chapter 2, we evaluated several non-viral transfection methods; both on human fetal tissue-derived SCs, as well as on human pluripotent stem cell-derived SCs. There was a marked difference in the requirements for transfection between the cells evaluated. We developed a linear poly(ethyleneimine)/DNA polyplex transfection protocol utilizing a DMSO shock to reach high transfection efficiencies in human immortalized SCs. This however did not translate directly into the stem cell-derived SC precursor-like cells, where cytotoxic responses were seen in several batches of cells. Screening of multiple poly(beta-amino ester)/DNA-based polyplexes did not improve cell health or survival. Based upon previous studies identifying anti-viral responses to cytoplasmic DNA[172,228-233], we synthesized mRNA with modifications to mask it from the host innate immune response, with improved transfection results and ability to perform serial transfections (two sequential tested) with maintained transfection efficiency. Collectively, these results indicate that the cytotoxic response was not dominated by the polymer carrier used, but rather through an anti-viral response to the DNA, and that efficient non-viral transfection could be achieved in human pluripotent stem cell-derived SC-like cells through the use of modified mRNA polyplexes.

Specific Aim 2: Determine the effects of key transcription factors on myelination using lentiviral constructs

In Chapter 3, we present studies testing if overexpression of key transcription factors known to control the SC maturation and myelination process can enhance the myelination frequency. We identified a transcription factor that directly increased the myelination frequency in primary rat SC/DRG neuron co-cultures. Functionalization of

human stem cell-derived SCs with the same factor caused cell death. Co-expression of the anti-apoptotic protein Bcl-xL alleviated this, although in only one batch tested so far, but the transcription factor overexpression failed to regulate known target genes despite robust transgene overexpression. These results suggest that while such an overexpression has an effect in the context of primary SCs, the stem cell-derived cells do not have the underlying genetic regulation in place for the transcription factor to exert its function, at least not in the cell preparation tested.

Specific Aim 3: Characterize the effects of cell functionalization on peripheral nerve injury regeneration

In vivo experiments using functionalized primary SCs seeded into nerve guides are currently under way to determine if the *in vitro* results can translate into *in vivo* enhanced myelination frequencies in a sciatic nerve injury model in mice. Nerve histomorphometrical evaluation will also be used to assess if it also may lead to an enhanced regenerative outcome, although the defect size possible in the immunodeficient mice may be too small to discern direct functional consequences.

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CHAPTER 2 - NON-VIRAL GENE DELIVERY VECTORS FOR SCHWANN CELL TRANSFECTION

2.1 Polyplexes and lipoplexes as DNA and RNA transfection carriers

Plasmid DNA and mRNA constitute non-viral options with very low or no risk of stable genomic integration, respectively, thus alleviating clinical safety concerns from long-term transgene overexpression and insertional mutagenesis. Naked or unprotected plasmids or mRNA molecules are however taken up by cells very inefficiently. Both polyplexes and lipoplexes, that is carriers based upon cationic polymers and lipids respectively, enhance cellular uptake and transfection efficiencies by complexing with the DNA/mRNA[1].

For *in vitro* transfection and cellular entry, the plasmid or mRNA needs to pass several barriers. First is to cross the cell membrane, which is rich in heparin sulfate proteoglycans and has a net negative charge, as do the polynucleotides[1]. Lipid carriers typically consist of a neutral linker connecting a positively charged head group and a hydrophobic anchor[2], while polymeric carriers have a positively charged backbone[1]. The positive charge complexes with the negative charge of plasmid DNA or mRNA, abolishing repulsive charge effects and reducing its size that can be up to micrometer size for plasmids[1], down to typically 150 – 600 nm for lipoplexes[2] or 30 – 100 nm for polyplexes[1]. In doing so, the complex acquires a net neutral or slightly positive surface charge that facilitates interaction with the cell membrane[3]. Particles of this size may be taken up by endocytosis or macropinocytosis[1,4,5]. Both plasmids and mRNA then need to

escape the endosome, liberate themselves from the carrier, and plasmids also need to enter the cell nucleus. The exact order or mechanisms by which these steps occur are not well understood. Lipoplexes are believed to fuse with the endosomal vesicle membrane, transferring the cargo through a flip-flop[6]. Polyplexes, such as those based upon poly(ethyleneimine) (PEI), have been proposed to exit through the so called "proton sponge effect"[7]. The theory is that the amines of the PEI buffer the acidic endosome, causing an influx of chlorine ions and an osmotic effect that causes the endosome to rupture. The validity of this model is however questionable. Theoretical calculations of endosomal membrane expansion from water influx appears insufficient to cause it to burst[8], pH changes seem to be limited[9], and imaging studies failed to observe any evidence of endosomal rupture[10]. For the case of plasmids, they further need to make it into the nucleus to be transcribed. Nuclear entry is greatly facilitated during cell division, as this is the only time that the nuclear membrane integrity becomes compromised. Otherwise molecules above 9 nm in size will have to go through an active transport via the nuclear pore complex[1]. The localization of the endosomal release also plays a role. Release of plasmid DNA in the perinuclear space can increase transfection efficiency, as intracellular diffusion of plasmid DNA appears limited[11-13].

The chemical makeup of nanoparticles will impact both their uptake, trafficking and decomplexation patterns. One of the most efficient polymeric transfection reagents is PEI, but it is also associated with cytotoxic response in cells[14]. With all nanoparticle formulations remaining highly inefficient in trafficking, only a few percent able to escape endosomes[4,10], the majority of the cargo that enters the cell fails to produce gene expression. The rest may cause cytotoxic responses in the cells through a number of

mechanisms reviewed by Xue *et al.*[14] and Parhamifar *et al.*[15], including induction of reactive oxygen species by cationic materials, interactions with and disturbance of intracellular compartments, and a limited biodegradability. As long as the intracellular trafficking and carrier release remains largely inefficient, carriers that exhibit efficient cellular entry will be correlated with the negative effects of doing so. With very limited understanding of the process, we have but a few tools at our disposal to design carrier properties with predictable outcomes. One such approach tackles both the toxic effects associated with polycations, and the disassembly of the carrier to liberate the cargo, namely design of degradable backbone moieties. This can for instance be done by incorporation of disulfide bonds, leveraging the more reducing conditions upon cellular entry caused by the presence of cellular glutathione[16-18].

In addition to the cytotoxic effects from the carrier, the payload itself may cause antiviral responses compromising cell health. Cytoplasmic presence of DNA[19] or non-self RNA[20] is an indication of a potential viral infection, causing a cellular response ranging from release of signaling molecules to apoptosis[21-23]. A number of mechanisms by which cells sense such DNA or RNA have been identified, as reviewed by Wu and Chen[23]. While the Toll-like receptors (TLRs) that sense DNA or RNA in the endosomes are predominantly present in immune cells, TLR-independent cytoplasmic sensors act within the cytosol of somatic cells. These sensors may distinguish between self and non-self nucleic acids through foreign structures or motifs, or incorrect subcellular localization of the nucleic acids. Consequently, transfection efficiencies can be improved by removal of such motifs, or by mimicking nucleoside and end modifications preventing immune recognition. For instance, plasmid transfection efficiencies can be enhanced by

removal of the bacterial backbone components resulting in what are known as minicircle plasmids[24,25], and mRNA transfection efficiency can be enhanced by removal of 5' triphosphates[26] and incorporation of modified nucleosides[27-29]. Interestingly, two of the workhorses in *in vitro* transfection studies, the HEK 293T and HeLa cells, were found to lack an RNA polymerase III-independent pathway for sensing foreign DNA, and mouse embryonic fibroblasts lose this pathway after immortalization and serially passaging through yet unidentified mechanisms[30]. This may in part explain differences in transfection efficiencies seen between immortalized cell lines and primary cells.

2.2 Enhanced plasmid transfection efficiency in human immortalized Schwann cells by DMSO shock

In order to develop efficient methods for plasmid transfection of SCs, we investigated if a brief exposure to a DMSO could improve transfection over that of the polyplex alone. We used linear PEI with a number average molecular weight of 7 kDa, as PEI is a well characterized and broadly used polymer for transfection already employed in clinical trials[31], and linear PEI with relatively low molecular weights have been shown to induce a less cytotoxic response than branched or high molecular weight counterparts[14,15]. After a preliminary screen of DNA dose and DMSO exposure tolerance on human fetal tissue-derived SCs, DNA conditions with DMSO concentrations of 25% - 30%, 1 – 2 min shock duration, 0.1 – 0.3 μg/cm^2 of DNA, and a 4-hour incubation time prior to DMSO application were selected according to Table 1 for further investigation using a GFP expression vector.

Characterization of the fluorescence intensity of the transfected cells by flow cytometry 17 hours post-shock revealed a greatly enhanced transfection efficiency of the GFP expression vector when DMSO shock was applied (Figure 2-1). Transfection without DMSO application was at a very low level (0.9% of cells, Figure 2-1E), while all groups subjected to DMSO treatment had >80% of the cells positive for GFP, giving almost an order of magnitude enhancement in yield of transfected cells. The highest transfection efficiency observed was 96.4 ± 2.0% (n = 12) of the cells under condition IV (Figure 2-1C, Table 2-1).

The cell health was evaluated through a WST-1 metabolic activity assay, with an untreated control serving as the reference metabolic activity level. Conditions I-III did not show a significantly reduced metabolic activity at 17 hours post-shock relative to the cells not receiving a DMSO treatment (Figure 2-1F), indicating that the cells maintained a good viability. Condition IV, which also had the highest transfection efficiency, however displayed a significantly reduced metabolic activity relative to all conditions except III. The general trend was for conditions displaying a higher average GFP intensity to also produce a reduced metabolic activity.

Of note is that while all the DMSO-treated conditions had similar yield of transfected cells, the average fluorescence intensity was dependent on the transfection conditions. Comparing conditions I vs. II shows an increased transgene expression level between DNA doses of 0.1 $\mu g/cm^2$ and 0.3 $\mu g/cm^2$ under otherwise identical conditions, indicating that a dose saturation point had not been reached at least for 0.1 $\mu g/cm^2$ DNA dose. As condition III, employing a lower DMSO concentration than condition II, showed a higher GFP expression level but lower (although not statistically significant)

metabolic activity suggests that the immortalized SCs tolerate the DMSO range used, and that toxicity may be more related to the transfection efficiencies produced. This is in agreement with the trends observed across the groups compared. Thus, the combined effects, not the mere exposure to DMSO or nanoparticles alone, dictate the impact on the cell health. Similar observations were made when tested on human fibroblasts, but these cells much more sensitive to the cytotoxic effects of the transfection (Figure 2-2).

To further study the applicability of the transfection method and observed transgene expression control, conditions I and III were selected for transfection of immortalized SCs with a pleiotrophin expression vector carrying a C-terminal GFP tag. These conditions using DMSO shock produced the biggest difference in transgene expression level, without significantly compromising cell viability (Figure 2-1E-F). Pleiotrophin (PTN) is a neurotrophic and neurotropic factor that exerts its function predominantly on motor neurons[32]. Functionalization of SCs to secrete PTN may therefore be a strategy to promote and guide motor axon regeneration. Secreted PTN in media conditioned over transfected SCs between 24 – 48 hours post-transfection was analyzed by dot blot, yielding 1.8 ± 0.1 ng/µl under condition I, and 2.7 ± 0.2 ng/µl under condition III, displaying a significant difference in expression level and confirming the previously observed control over transgene expression level (Figures 2-3 and 2-4). Analysis of conditioned media by fluorometry confirmed that the transgene expression level could be controlled by tuning the transfection conditions (Figure 2-5).

Collectively, these results show that DMSO shock can enhance transfection efficiencies of PEI/DNA polyplexes, and that tuning transfection conditions also affect transgene expression levels. This was observed for both human immortalized SCs, as

well as primary human fibroblasts, although the latter showed a greater sensitivity to transfection. While it has previously been reported that DMSO shock can enhance transfection efficiencies for other cationic carriers, such as DAEA-dextran[33], polybrene[34,35], calcium phosphate[36], and poly(L-ornithine)[37,38], none have reported transfection efficiencies as high as those we observed employing DMSO shock with PEI/DNA polyplexes. As they all used different cell lines for their studies, direct comparisons cannot be made due to cell type dependence. We can however conclude that DMSO shock works very well in significantly enhancing DNA transfection efficiencies for PEI/DNA polyplexes. None of the studies, ours included, can identify which step in the plasmid trafficking to the nucleus that is enhanced. A likely candidate is the endosomal escape, based upon the fact that this step has been seen as highly inefficient in imaging studies[4,10], and that direct passage through the cell membrane may not effectively bring the particles to the perinuclear space for subsequent nuclear entry[11-13]. It should however be noted that observation from lipoplexes suggest that DNA labeling can affect binding and decomplexation from the carrier, and therefore studies using labeled particles may not fully recapitulate the trafficking of non-labeled particles and plasmids[39].

The cell-type dependence on transfection efficiency can have several explanations, including different uptake mechanisms, mitotic activity, and transcriptional and translational activity. Of note was however the sensitivity to transfection between immortalized SCs and primary fibroblasts used in our experiments, where immortalized SCs appeared much more tolerant than the fibroblasts. While sensitivity to the nanotoxic effects discussed in the previous section could vary, an interesting thought is that they

may have different levels of sensitivity and response to cytoplasmic DNA. As mentioned previously, Chiu *et al*. found that mouse embryonic fibroblasts lost the ability to detect foreign DNA through the RNA polymerase III-dependent pathway upon immortalization and serial passaging[30]. It is therefore possible that such effects could have rendered the immortalized SCs more amenable to transfection.

2.3 Transfection of human pluripotent stem cell-derived Schwann cell precursors

Plasmid transfection conditions were screened for human pluripotent stem cell-derived SC precursor-like cells derived in the lab of Dr. Gabsang Lee. Transfection with plasmid DNA/PEI polyplexes and DMSO shock never produced conditions with reproducible results regarding cell survival and health, frequently wiping out entire wells of cells (data not shown). Due to the high cytotoxicity observed, we screened a library of poly(β-amino esters)[40] (PBEAs) to find carrier chemistries that may overcome the cytotoxic response. While no DMSO was necessary to achieve good transfection efficiencies (Figures 2-6 and 2-7), the cells transfected displayed unhealthy morphologies at two days post transfection, which escalated as the dose was increased from 300 ng to 600 ng in a 96 well plate format. Figure 7 shows the best performing group for transfection yield while maintaining a relatively healthy morphology. The transfections were however not compatible with serial transfections (data not shown), and the significantly reduced cell health with increased dose to 600 ng and only 40% transfection efficiency at 300 ng would render very low co-transfection efficiencies of multiple transcription factors at doses tolerable by the cells.

With such a wide range of carrier chemistries failing to alleviate the cytotoxic response, we asked whether or not it may be due to an antiviral response to the foreign DNA introduced rather than to the polymer carrier itself. It has previously been reported that masking the mRNA from innate immune responses by nucleoside modifications has facilitated cell reprogramming[41]. The modified mRNA transfection of GFP allowed for serial transfection using either PEI or PBEAs containing reducible di-sulfide bonds in their backbones as carriers, with dose-dependent transfection efficiency in SC precursor-like cells. A control transfection using PEI and DMSO shock did not display the same dose response nor any increase in transfection yield upon serial transfection (Figures 2-8 and 2-9). The cells also appeared overall healthier after transfection with modified mRNA than plasmid (data not shown). These results suggest that the cytotoxic response and limited transfection capability of hESC-derived SCPs was due to, at least in large part, an anti-viral response to cytoplasmic DNA. We do note that the results should be verified on multiple batches of stem cell-derived cells. However, as the same batch of cells had a detrimental response to plasmid transfection, but retained a healthy morphology and growth after mRNA transfection, we believe that it is a likely explanation for our previous observations as well, and due to cost and time constraints moved the focus of our experiments to determining a combination of transcription factors that can functionalize the cells into a myelinating phenotype (see next chapter), intending to return to the question after a transcription factor combination has been identified.

2.4 Figures

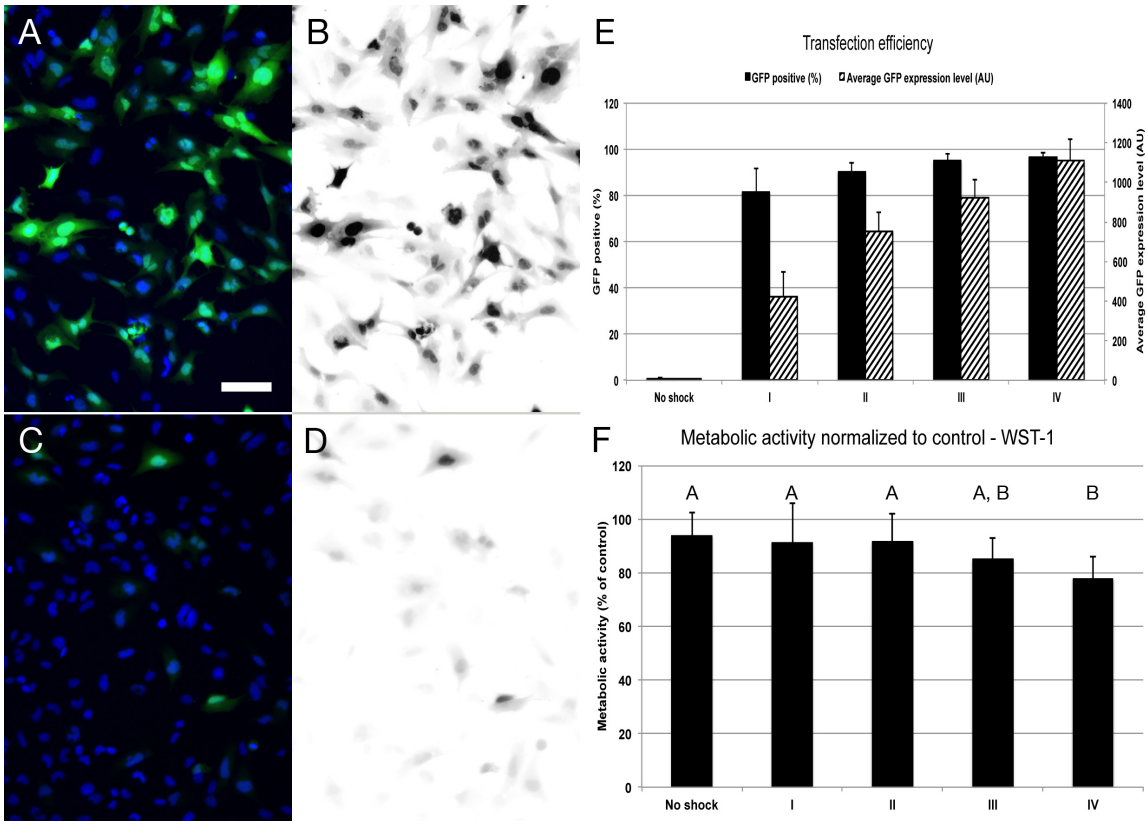

Figure 2-1 DMSO shock enhances transfection efficiency of PEI/DNA nanoparticles in human immortalized SCs.

Images of transfected Schwann cells under conditions IV (A, B) and I (C,D), conditions in Table 2-1. GFP signal in green with nucleus (DAPI) in blue (A, C). GFP signal depicted in black on white background (B, D). Scale bar = 100 μm. Bar graphs of average GFP expression level on left scale and % positive cells on right scale (E). Average expression level was significantly different between all groups ($p < 0.01$, n=12, Ryan-Einot-Gabriel-Welsch test). Metabolic activity relative to untreated control with homogenous subsets denoted by letters ($\alpha = 0.05$, n=12, Games-Howell test) (F). Error bars = one standard deviation.

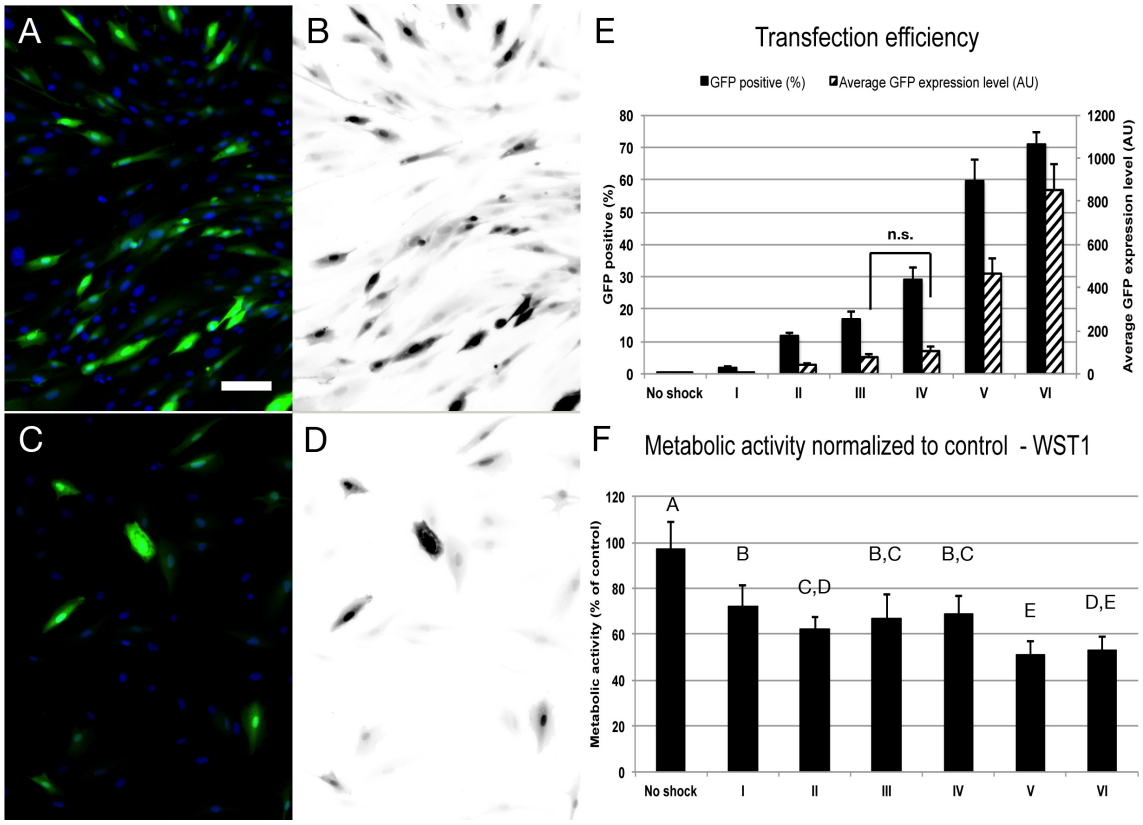

Figure 2-2 DMSO shock enhances transfection efficiency of PEI/DNA nanoparticles in human primary fibroblasts.

Images of transfected fibroblasts under conditions V (A, B) and IV (C,D), conditions in Table 2-2. GFP signal in green with nucleus (DAPI) in blue (A, C). GFP signal depicted in black on white background (B, D). Scale bar = 100 μm. Bar graphs of average GFP expression level on left scale and % positive cells on right scale (E). Average expression level was significantly different between all groups but III and IV, marked n.s. ($p < 0.01$, $n = 10$, Ryan-Einot-Gabriel-Welsch test). Metabolic activity relative to untreated control with homogenous subsets denoted by letters ($\alpha = 0.05$, Games-Howell test) (F). Error bars = one standard deviation.

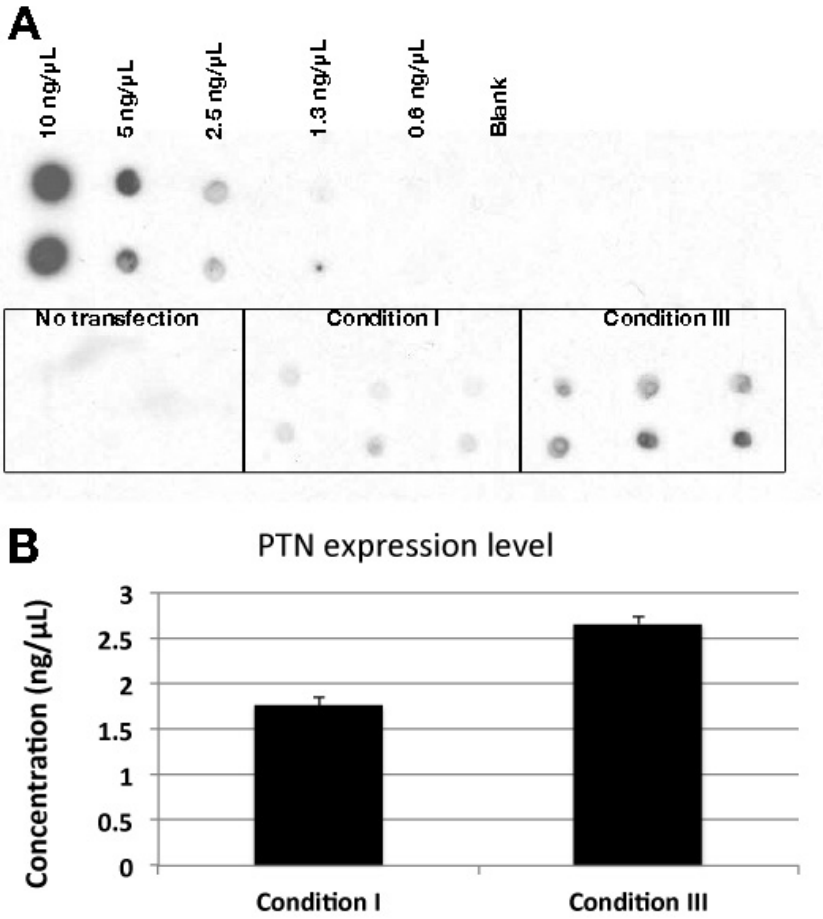

Figure 2-3 Tunable transgene expression level of PTN in supernatant of transfected human immortalized SCs.

PTN levels in supernatant conditioned by immortalized Schwann cells 24 – 48 hours post-transfection under conditions I and III (Table 2-1) with a PTN expression plasmid. Dot blot quantification PTN confirmed the controllable expression level (A). Quantification relative to a standard dilution series of human recombinant PTN yielded 1.8 ± 0.1 ng/µl under condition I, and 2.7 ± 0.2 ng/µl under condition III, displaying a significant difference in expression level (B, concentrations of 10 ng/µl – 1.3 ng/ µl used for regression, lower dot of 1.3 ng/ µl excluded, $R^2 = 0.989$, $p = 0.02$, Student's independent two-tailed t-test, $n = 3$). Error bars = one standard deviation.

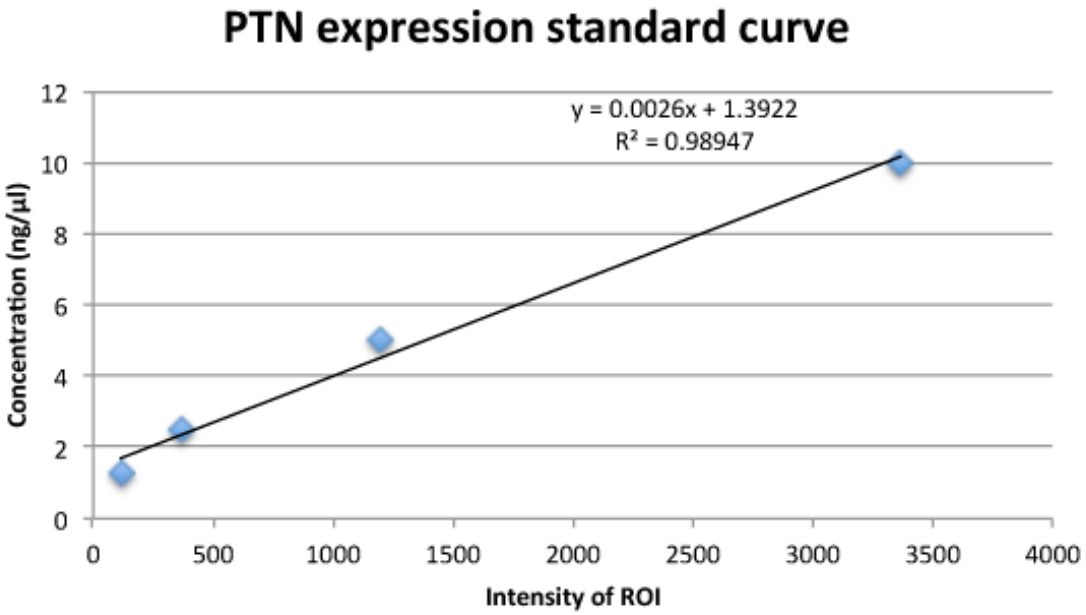

Figure 2-4 PTN dot blot standard curve.

ROI intensity value was determined as the average between the two dots at each concentration (Figure 2-3A).

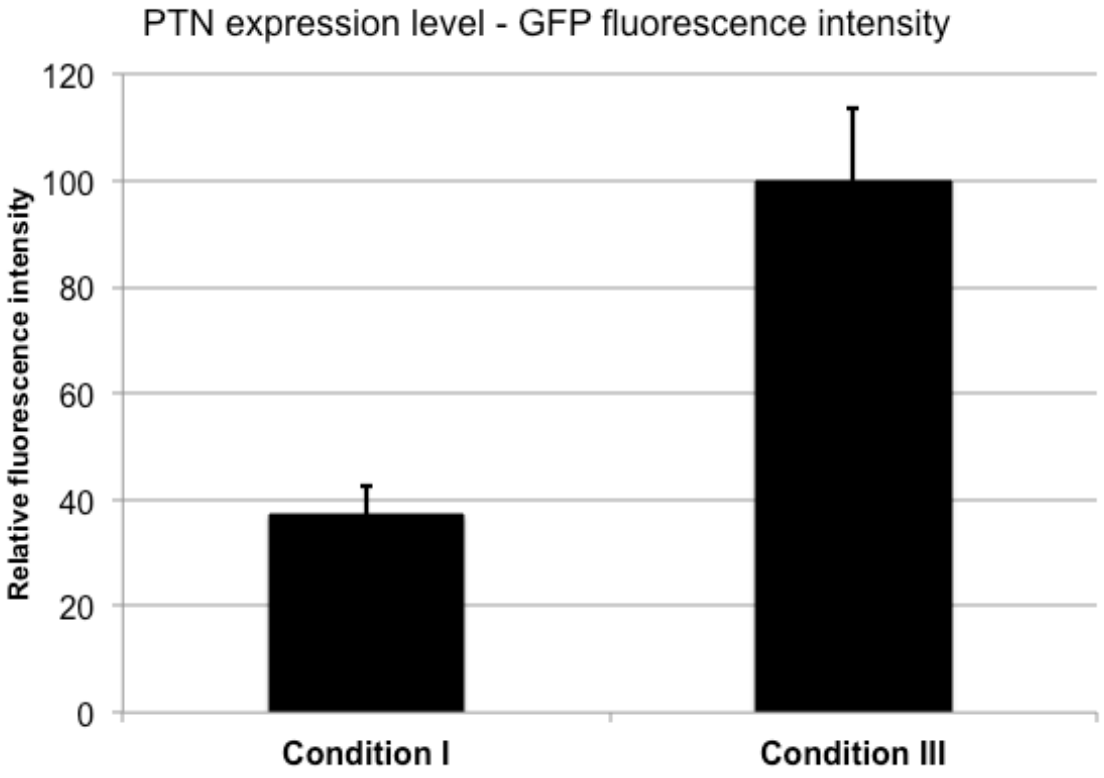

Figure 2-5 Controllable expression level of PTN as determined by fluorometry.

PTN level in supernatant of transfected human immortalized SCs under conditions I (n=3) and III (n=4) according to talbe 2-1. Values are normalized to that of the highest average expression. The difference was statistically significant (α=0.0005, one-sided independent t-test). Error bars = one standard deviation.

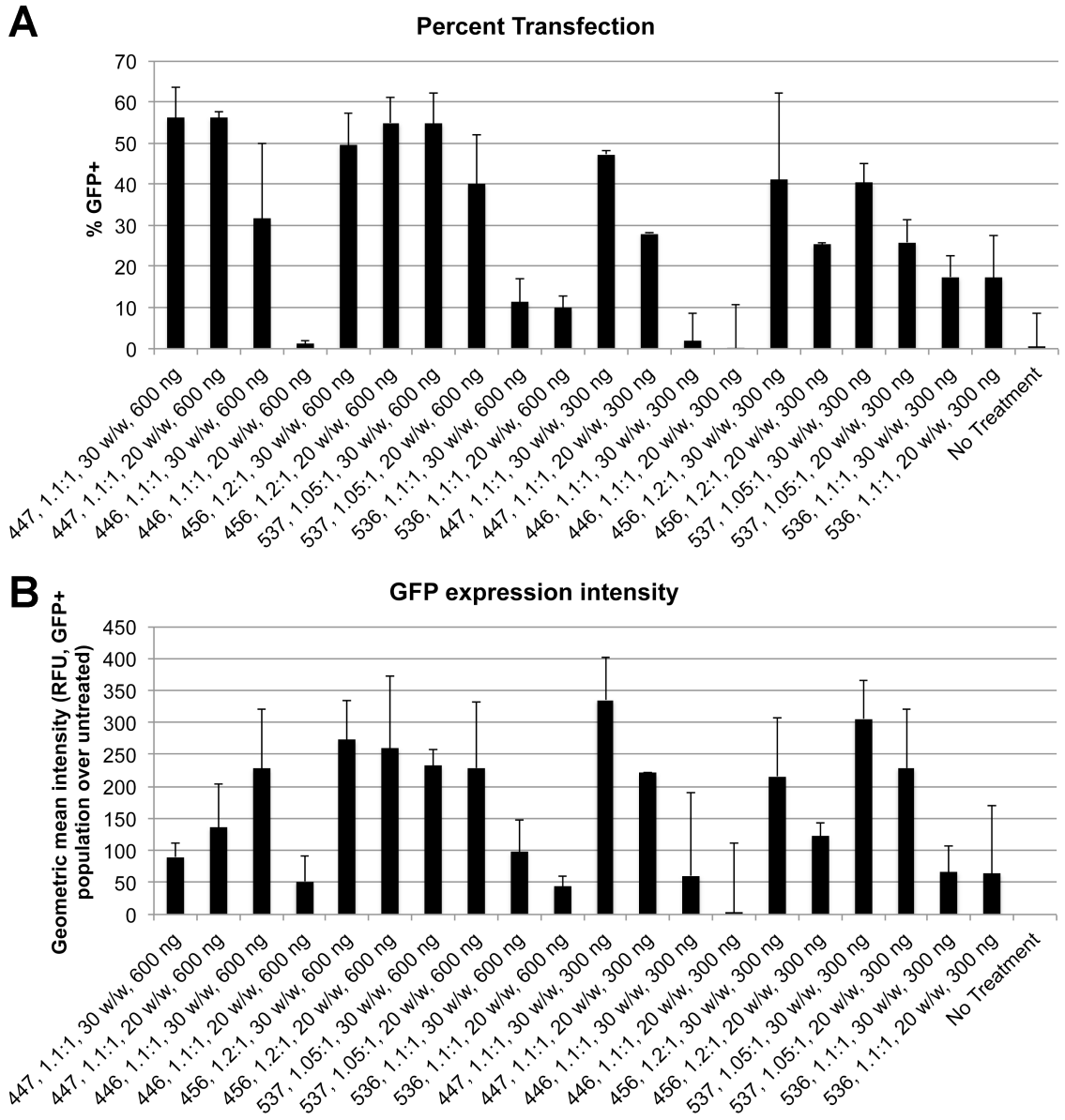

Figure 2-6 Screening of PBAE carriers for plasmid transfection of human pluripotent stem cell-derived SCP-like cells.

(A) Percent transfected as determined by flow cytometry. (B) GFP expression intensity (FL-1 channel) above that of non-transfected cells. Carrier names as described in Materials and Methods. n = 4 per gourp. Error bars = one standard deviation.

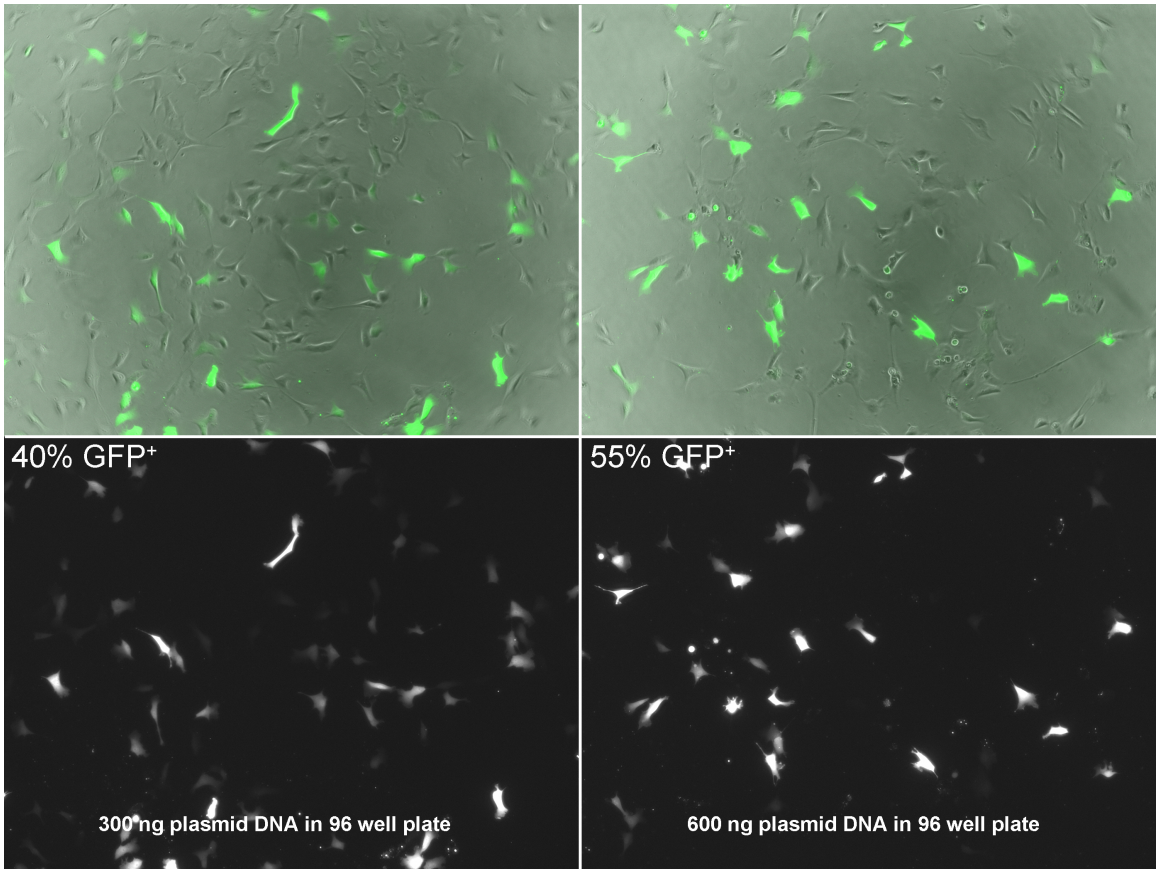

Figure 2-7 PBAE/plasmid polyplex transfection is limited by cytotoxic effects in human pluripotent stem cell-derived SCP-like cells.

Human embryonic stem cell-derived Schwann cell precursor-like cells transfected with PBEA 537 and GFP expression plasmid at 300 ng (left) or 600 ng (right) DNA per well in a 96 well plate format, imaged at two days after transfection. Images represent the best performing group of PBEA carriers tested. Top row shows bright field image with GFP overlayed, and bottom shows GFP signal only in white on a black background. Percent positive cells as determined by flow cytometry are indicated in the top left corners.

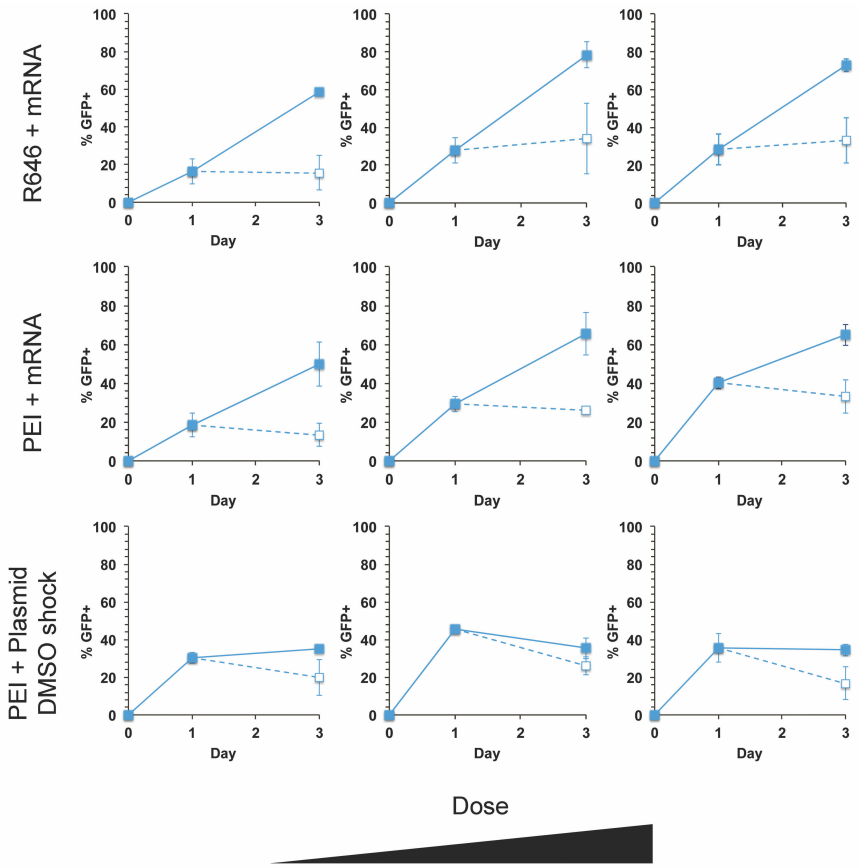

Figure 2-8 Modified mRNA transfection produces efficient serial transfections with a good dose-response compared to plasmid transfection with DMSO shock.

hESC-derived SCPs were transfected with either mRNA (A-H) or plasmid (G-I) at day 0 (open dots) or day 0 and 1 (solid dots). (A-C) PBAE R646 carrier with (A) 100 ng, (B)150 ng or (C) 200 ng of EGFP mRNA (D-F) L-PEI 17kDa carrier with (D) 50 ng (E) 100 ng or (F) 150 ng of EGFP mRNA (G-H) L-PEI 7kDa carrier using DMSO shock at (G) 50 ng (H) 100 ng or (I) 150 ng EGFP plasmid under CMV promoter. Error bars = average ± standard deviation (n=4).

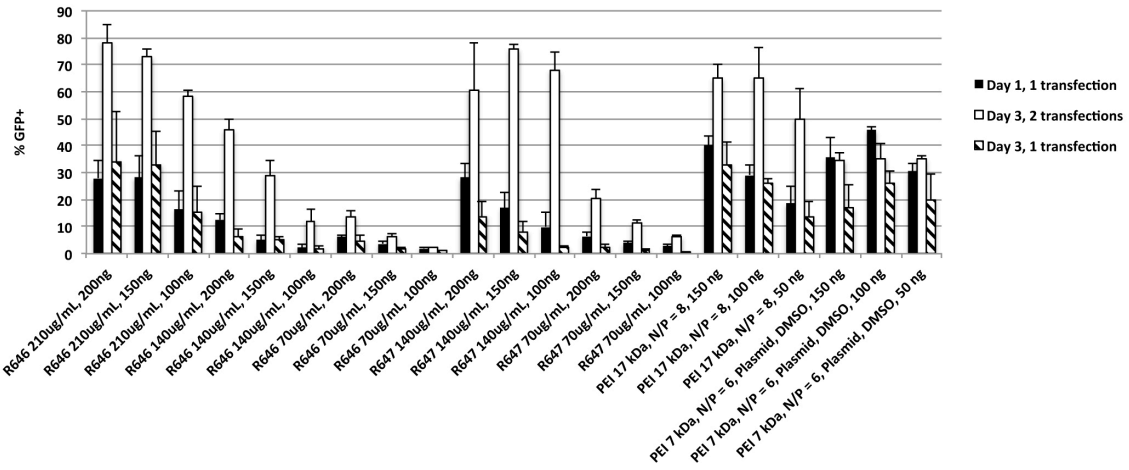

Figure 2-9 Transfection yield of complete data set for screening of mRNA transfection carriers on hESC-derived SCPs.

Carrier annotations as described in Materials and Methods. n = 4 per group and time point. Error bars = one standard deviation.

2.5 Tables

Schwann cell transfection conditions			
Group	**DNA concentration (μg/cm^2)**	**DMSO concentration (wt.%)**	**Shock duration (min)**
No shock	0.30	N/A	N/A
I	0.10	30	1
II	0.30	30	1
III	0.30	25	1
IV	0.30	30	2

Table 2-1 Schwann cell transfection conditions.

The conditions varied are listed. DNA incubation time was 4 hours for all groups.

Fibroblast transfection conditions		
Group	**DNA concentration (μg/cm^2)**	**DMSO conc (wt. %)**
No shock	1.00	0
I	0.25	20
II	0.50	20
III	1.00	20
IV	0.25	30
V	0.50	30
VI	1.00	30

Table 2-2 Fibroblast transfection conditions.

The conditions varied are listed. The DNA incubation time was 4 hours and the shock duration 1.5 min for all groups.

2.6 Materials and Methods

2.6.1 Plasmids and nanoparticle formation

gWiz-GFP plasmid was purchased from Aldevron (Fargo, ND, USA). Plasmid for GFP-Myc-His-tagged human pleiotrophin (PTN) was produced by cloning cDNA for human PTN into pCDNA 3.1. The nanoparticles were formed from a 100 μg/ml solution of plasmid DNA in water, to which an equal volume of linear PEI, MW = 7,000 kDa (Polymer Chemistry Innovations, Inc., Tucson, AZ, USA), as confirmed by gel permeation chromatography, was added by vigorous pipetting to achieve an N/P ratio of 6.

2.6.2 Human fetal tissue-derived immortalized Schwann cells: origin, culture and transfection protocol

The immortalization and characterization of human fetal tissue-derived Schwann cells has been described elsewhere[42]. The cells were maintained at 37^{o}C, 5% CO^2 in air, in a medium consisting of 10% (v/v) FBS (Gibco, Grand Island, NY, USA), 2% (w/v) D-(+)-glucose (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM GlutaMAX™ (Gibco), 1X B-27 Supplement (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Quality Biological, Gaithersburg, MD, USA) in 1X Neurobasal medium (Gibco). Cells were passaged to an expansion ratio of 1 to 6 using TrypLE™ Express (Gibco) when reaching approximately 90% confluence.

Cells were plated at ~10,000 – 12,500 live cells/cm^2 as determined by trypan blue exclusion (Invitrogen, Grand Island, NY, USA) and Countess cell counter (Invitrogen) at

default settings. Transfection was performed the next day at approximately 30% confluence. Prior to the DMSO shock, Schwann cells were incubated with DNA nanoparticles for 4 hours at 37^{o}C, 5% CO^{2}. The shock was performed by aspirating the cell culture media, completely covering the cells with a solution of DMSO (ACS grade, Sigma-Aldrich) in DMEM (Mediatech, Manassas, VA, USA), aspiration, followed by a wash using DMEM before applying the cell culture medium.

Varying concentrations of DMSO, DNA density and shock duration were used according to Table 1. The shock duration was defined as the time from the application of DMSO solution until the cells received DMEM in the washing step.

2.6.3 Fibroblasts: origin, culture and transfection protocol

Human neonatal dermal fibroblasts isolated from foreskin (American Type Culture Collection, Manassas, VA, USA, catalog number PCS-201-010) were maintained in DMEM with 4.5 g/l glucose, L-glutamine and sodium pyruvate (Mediatech) supplemented with 15% (v/v) fetal bovine serum (Gibco) and fibroblast growth factor 2 (Peprotech, Rocy Hill, NJ, USA), using a 1 to 6 expansion ratio when cells reached near-confluence. At day 12 in culture, cells were plated at a density of 20 000 live cells/cm^{2}, as determined by trypan blue exclusion (Invitrogen) and Countess cell counter (Invitrogen) at default settings. Transfection was carried out the following day.

The transfection was performed as described for the Schwann cells. DNA and DMSO concentrations were varied according to Table 2-2.

2.6.4 Transfection efficiency and cell viability assessment

17 hours post shock, Schwann cells for gene expression quantification were detached by TrypLE™ Express (Gibco), and an equal volume of neutral buffered 10% formalin solution (Sigma-Aldrich) was added to fix the cells. The GFP transfection efficiency and average fluorescence intensity was determined by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson, Heidelberg, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm (FL-1). Signal amplification voltages were adjusted before each set of measurements not to saturate the scale for the brightest group, with absolute values reported in the graphs. Positive cells were defined as those with an expression level above which <0.01% of the control cells were found. Comparisons were made between cells of the same passage that were transfected and analyzed in the same session to minimize methodological errors.

Cells for fluorescent imaging were fixed for 20 minutes using 10% neutral buffered formalin (Sigma-Aldrich) and stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 10 minutes, with three washes in PBS after each step. Cells were imaged with a Nikon Eclipse TE2000-E inverted microscope using Nikon CFI Plan Fluor 10x and 20x objectives with N.A. of 0.30 and 0.45 respectively, as well as NIS Elements software (Nikon Instruments Inc., Melville, NY, USA). Contrast was equally adjusted for all images with reference to a non-treated sample to eliminate autofluorescence. Black and white images were obtained by displaying the GFP signal in white using NIS elements, followed by a color inversion using Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA). This was done to better visualize cells displaying a low expression level.

Metabolic activity was assessed by a WST-1 assay (Roche Applied Science, Indianapolis, IN, USA) as an indication of the cell viability. Assay was performed according to the manufacturer's instructions, with volumes adjusted for 48-well plates. The absorbance at 450 nm was measured after 1 hour using a Tecan NanoQuant Infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). Metabolic activity was depicted as normalized to the value obtained for the untreated control. Fibroblasts were analyzed the same way 24 hours post-shock.

2.6.5 Pleiotrophin expression level analysis

The transgene expression level of pleiotrophin was assessed by two assays. First, PTN concentration in the supernatant from transfected SCs was assessed via the GFP fusion tag. For these experiments, the medium was prepared out of phenol red-free Neurobasal medium (Gibco). The medium was exchanged 24 hours post-transfection, and subsequently collected for analysis 24 hours later. Measurements were performed using a FluoroLog 3-22 fluorometer (HORIBA Jobin Yvon Inc., Edison, NJ, USA) with samples in plastic cuvettes. Emission intensity of the supernatant from cells transfected under conditions I and III (Table 1) was collected from 495–700 nm with a 5 nm slit size, using a 480±5 nm excitation wavelength. Comparison between conditions was performed on the peak emission value after smoothing using a lo-pass FFT-filter with a 0.1 (nm)-1 cutoff, and subtraction of the emission from the supernatant of non-transfected cells using Origin 8 (OriginLab, Northampton, MA, USA).

For a quantification of the secreted amount of PTN, cells were treated as for the above fluorometer assay, but first using regular Neurobasal medium (Gibco) followed by

OptiMEM® (Gibco) 24 hours post-transfection. 24 hours later, the supernatant was collected, a protease inhibitor cocktail (Sigma-Aldrich) was added and samples stored at -20^{o}C until the time of analysis. A standard dilution series was made from recombinant human PTN expressed in E.Coli (Sigma-Aldrich). The lyophilized powder was reconstituted in PBS with added protease inhibitor cocktail (Sigma-Aldrich) and the stock concentration was verified by BCA protein assay (Pierce, Rockford, IL, USA) according to manufacturer's protocol with a BSA reference performed in triplicate. The stock was then diluted to concentrations of 10 ng/µl, 5 ng/µl, 2.5 ng/µl, 1.3 ng/µl and 0.6 ng/ µl plus blank.

The dot blot was performed using 1 µl of sample per dot onto a nitrocellulose membrane (BioRad, Hercules, CA, USA), applied in two drops. Blocking was performed over night at 4^{o}C in 5% non-fat milk in TBST (BioRad). Membranes were then incubated with anti-pleiotrophin (Sigma-Aldrich, P3743) at 0.5 µg/ml in 5% non-fat milk in TBST at room temperature for 1 hour, washed five times in TBST, reacted with HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA, SC-2020) for 2 hours at 0.4 µg/ml in 5% non-fat milk in TBST at room temperature for 1 hour, followed by five washes in TBST at room temperature. Development was done using Amersham ECL Prime Western Blotting Detection Reagent and an enhanced chemiluminescence ECL Western Blotting Analysis System (GE Healthcare/Amersham Biosciences, Piscataway, NJ, USA).

The dot blot intensity was analyzed using ImageJ 1.46r (NIH, Bethesda, MD, USA) by first subtracting the background followed by intensity analysis of circular regions of interest centered at the dot with the area kept constant. Using the standard

titration curve, the stain intensity was used to estimate the secreted concentration of PTN in the supernatant. The standard concentrations of 10 ng/µl, 5 ng/µl, 2.5 ng/µl and 1.3 ng/µl (R^2 = 0.989) were used, while 0.6 ng/µl was excluded as it was outside the linear range with the relevant exposure time, and the lower dot at 1.3 ng/ µl was excluded due to a mark on membrane (Figure 2A).

2.6.6 Transfection of human embryonic stem cell-derived Schwann cell precursor-like cells

Schwann cell precursor-like cells were derived from human embryonic stem cells in the lab of Dr. Gabsang Lee (Johns Hopkins University) according to a yet unpublished proprietary protocol. The cells were provided as pre-seeded plates with media aliquots for use with the transfection.

In vitro transcription (IVT) was performed as described by Warren *et al.*[41] with slight modifications. 990 ng plasmid template was used for every 20 µl reaction, and 5' capping was performed enzymatically using a ScriptCap m^7G capping reaction (CellScript, Madison, WI, USA) according to the manufacturer's instructions after the DNAse treatment, as opposed to inclusion of an ARCA cap analog in the IVT reaction as done by Warren *et al*. The product was purified on MEGAclear columns (ThermoFisher, Waltham, MA, USA), eluted into nuclease-free water and the concentration adjusted to 100 ng/µl and stored as single use aliquots at -80^{o}C.

PBAEs were synthesized and named as previously described for plasmids[43] and RNA[44]. For the plasmid transfection PBAE synthesis, ratios of backbone and side chain monomers are indicated (Figure 2-6). For instance, "447, 1.1:1, 30 w/w, 600 ng"

indicates that backbone monomer 4, side chain monomer 4, and end group molecule 7 were used in the synthesis, with backbone to side chain monomer ratios at 1.1:1. The mass ratio between carriers to DNA was 30, and the total dose in the well 600 ng. For mRNA transfection, PBAEs were mixed with mRNA at a 1:1 mass ratio, while PEI was indicated by a nitrogen to phosphate molar ratio (N/P ratio) between carrier and mRNA. PBAEs have the final concentration in the media as indicated, as well as the total dose (Figure 2-9).

2.6.7 Statistical analysis

Statistical analysis was performed using SPSS Statistics 19 (IBM, Armonk, NY, USA). gWiz-GFP expression and metabolic activity was compared using MANOVA, Pillai's trace. Significant differences were followed up by ANOVA and Ryan-Einot-Gabriel-Welsch or Games-Howell post-hoc tests when applicable, for groups with or without equal variance assumed respectively. Equal variance was assessed by Levene's test. All tests were performed at a level of $\alpha = 0.05$. n = 12 samples per group for SCs, and n = 10 for fibroblasts.

The PTN concentrations in the supernatant from the dot blot assay were subjected to two-sided independent t-tests with equal variance assumed following Levene's test, both at a level of $\alpha = 0.05$ (n=3).

PTN expression in GFP-tagged fluorometer assay was compared by measured peak fluorescence intensity from the fluorometer readout, subjected to a one-sided independent t-test ($\alpha = 0.0005$) (n=3 for condition I and n=4 for condition III, according to Table 1). Equality of variance was determined by Levene's test ($\alpha = 0.05$).

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CHAPTER 3 - HARNESSING THE TRANSCRIPTIONAL REGULATION OF SCHWANN CELL MYELINATION FOR IMPROVING CELL FUNCTIONALITY

3.1 Introduction

The transcriptional regulation underlying Schwann cell (SC) maturation and myelination is still an active research area. A few key regulators have emerged that have served as candidate factors in our studies. As outlined in Chapter 1, SCs arise from the neural crest precursor cells. After maturation to the immature SC stage, the cells make a decision on whether to mature into myelinating or non-myelinating SCs. The myelinating SCs, which are the focus of this dissertation, go through a so-called radial sorting process to form a one-to-one relation with their axons (as opposed to oligodendrocytes in the central nervous system that myelinate multiple axons, or the non-myelinating SCs that support multiple small caliber axons without forming myelin), and take on a pro-myelin SC phenotype. These cells then proceed to wrap around the axon, forming a dense, multi-layered membrane structure from which most of the cytosol is extruded[1]. The myelin has a distinct makeup from the non-myelinating, activated, or immature SC membrane, and a feed-forward cascade of transcription factors drives the expression of the proteins that create and stabilize the myelin. So far, three of the transcription factors that directly bind to regulatory elements of myelin genes for their activation and expression under normal development have been identified, namely *Sox10*, *Oct6* (also known as *Pou3f1*, *Tst1*, or *Scip*), and *Egr2* (also known as *Krox20*)[2].

Sox10 is expressed throughout the different stages of SC development, starting at the migratory neural crest cell stage[3], and is required for SC specification. In SCs, it controls the expression of the neuregulin receptor *Erbb3*, important for SC migration, interplay between SCs and axons, and SC survival[4-11]. *Sox10* also takes part in the upregulation of *Oct6* through homodimeric binding to the SC enhancer (SCE)[12,13], constituting the first step of the feed-forward regulation, taking place at the pro-myelinating SC stage[14-16].

In the next step, *Sox10* and *Oct6* upregulate *Egr2* expression by acting upon a cis-regulatory element called the myelinating SC element (MSE)[17-19]. Mouse models deficient in *Oct6* show a transient arrest at the pro-myelinating stage, but this is rescued through *Brn2* expression, and myelination proceeds normally with a slight delay. Double mutants for *Oct6* and *Brn2* however have their SCs terminally arrested at the pro-myelinating stage, leading to hypomyelination[20].

For the formation of mature myelin to occur, *Oct6* needs to be downregulated[21], at least in part, by *Egr2*[22]. *Sox10* and *Egr2* continue to be expressed in the mature, myelinating SC, and are required for myelin maintenance[23,24].

While these factors constitute the known key orchestrators of SC myelination in the sense that they directly bind to the regulatory elements, they do not act alone. *Sox10* has been found to recruit the BAF remodeling complex[25] and HDAC1/2[26] in its regulation of the feed-forward cascade, and thus chromatin remodeling is likely required for the induction of *Oct6* and *Egr2*. Studies of *CnB1* mutant mice, lacking a key subunit for calcineurin signaling, revealed a critical link between intracellular calcium signaling

induced through neuregulin signaling, and the upregulation of *Egr2* and myelin protein zero expression through synergistic regulation by *Sox10* and *Nfatc4*. Thus, axonal signaling leads to dephosphorylation and nuclear entry of *Nfatc4*, resulting in myelin gene expression through cooperation between *Nfatc4* and *Sox10*[27] at the pro-myelinating SC stage. *Yy1* has also been shown to be a critical link in *Egr2* induction, and *in vitro* studies suggest that neuregulin signaling activates *Yy1* through posttranslational modification by MEK kinase activity to upregulate *Egr2* expression[28]. Additionally, the NuRD complex has been shown to be required for formation of myelinating SCs. In animals with a conditional deletion of one of the key components of the NuRD complex, *Chd4*, hypomyelination and radial sorting defects and a misexpression of genes related to an immature SC phenotype late in development (P30) was observed. The NuRD complex was found to be recruited to many of the *Egr2* target sites, thus suggesting a recruitment of NuRD by *Egr2*[29]. *Egr2* further depends on its interactions with *Nab1/2*. Mice lacking both factors phenocopy the *Egr2* deficient mice[30]. As the Nab proteins have been found to interact with *Chd4*[31] as well as *Egr2*[32], they have been proposed as a link for NuRD recruitment by *Egr2* to regulatory sites, and can act as both co-activators[33] and co-repressors[29,32]. *Sox10* also depends on the Mediator complex in its activation of *Egr2* expression. Conditional knockout of the subunit *Med12* in glia prevented myelination in both the PNS and the CNS. For SCs, *Med12* was found to be recruited to the MSE, and *Sox10*-induced activation of an *Egr2* reporter construct was dependent on Mediator complex subunits for its activation, suggesting that Mediator complex recruitment by *Sox10* to the MSE is required for myelin gene induction[34]. It is of interest to note the importance of *Sox10* in the specification and maturation of both SCs and

oligodendrocytes. As in SCs, *Sox10* plays a key role for cell specification as well as for terminal functional differentiation into myelinating oligodendrocytes, but the target genes it regulates are different[2,35]. With its ability to recruit remodeling complexes, it may act as a glial pioneer factor, making the chromatin accessible to other regulatory factors[2].

In addition to the factors responsible for upregulation of myelin genes, downregulation of inhibitory factors is necessary for the maturation of SCs and subsequent myelination. For instance, Notch signaling has been found to inhibit myelination, but also to promote maturation of SC precursors and promote their proliferation[36]. Additional negative regulators of myelination are *cJun*[37] and *Sox2*[38]. Interestingly, there exist a reciprocal regulation between these negative regulators and *Egr2*[36-38], suggesting that their respective expression levels may be able to regulate myelin gene expression. More recently, *Zeb2* has been identified as a transcription factor responsible for downregulation of *Sox2* and inhibiting Notch signaling by repressing *Hey2* expression. *Zeb2* mutants failed to downregulate inhibitors of myelination, and displayed radial sorting deficits[39,40]. The function of micro RNAs in SC differentiation is still not very well understood. Conditional knockout studies of *Dicer* have demonstrated their involvement in myelin regulation[41,42], and groups of micro RNAs show expression patterns suggesting different roles at the different stages of myelination. A link between gene regulation by micro RNAs and key SC transcription factors has further been suggested to occur through direct regulation of micro RNA expression by *Sox10*[43].

From the perspective of cell engineering and promoting maturation through overexpression of some key transcription factors, there are a few considerations to be made given the above discussion. First, there is a core unit of transcription factors that

constitute the feed-forward cascade to the extent that it is known today, namely *Sox10*, *Oct6*, and *Egr2*, with *Brn2* believed to serve a largely redundant role to *Oct6*[17,20]. The end-point of this cascade is the upregulation of myelin transcripts by *Sox10* and *Egr2*, as well as the downregulation of some key inhibitors. Second, these factors recruit other components of gene regulation and possibly epigenetic modification to their target sites, highlighting the fact that all such factors also need to be present for the full function to be manifested. Third, the timing of myelination also depends on downregulation of its inhibitors. The regulation of some of the key components necessary for this to happen, *e.g. Zeb2*, is not fully understood in the context of SC myelination, and it is therefore unknown if they are part of the same signaling axis that regulates the *Sox10*, *Oct6*, *Egr2* cascade, or if they require additional signaling cascades to be activated. What this means is that any derived human SC, be that through stem cell reprogramming, direct conversion or transdifferentiation, will have to have these additional components in place, as well as the capability to express receptors for functional axon-SC communication. The success therefore is highly dependent on the underlying cell preparation quality and resemblance to primary SCs, the factors necessary are likely differentiation stage specific, and the epigenetic regulation of chromatin accessibility likely to play a role for the binding of certain transcription factors for their gene regulation. This becomes apparent from the fact that these factors serve other functions and have other targets in other cell types, and dysregulated cells may have unintended or failed target gene regulations from the overexpression of these transcription factors. As pointed out above, *Sox10* serves similar functions in oligodendrocyte development as it does in SC development, but regulates, at least in part, other genes[2,34,35]. *Oct6* is involved in keratinocyte

development[44], *Egr2* in rhombomere patterning[45,46], and *Brn2* takes part in cortical layer II, III and V neuronal subtype specification[47,48] and has been used for neuronal induction in cell programming[49,50], just to name a few examples. To expect overexpression of a few key transcription factors to reach fully functional, myelinating SCs may therefore seem like a tall order. There is however some respite from the fact that some related transcription factors have the key regulatory domains conserved[19,51], and the gene regulatory network thereby has some plasticity in its composition. Also, as described in Chapter 1, this is one of the most common approaches used in cell reprogramming. It has also been used in some cases of subtype specification, *e.g.* for neuronal subtypes[52], and one may argue that myelination is the SC equivalent of subtype specification between myelinating and non-myelinating SCs.

In this chapter, we first present a set of experiments to determine if transcription factor overexpression can improve the myelination frequency from SCs. This was done through overexpression studies in primary rat and mouse SCs, thus constituting a model in which myelination for sure can occur. Our results suggest that overexpression of *Egr2* may indeed enhance myelination frequency even in primary SCs. We also present some preliminary results on human stem cell-derived SCs, although no successful functionalization has been observed. The fact that the cells did not show upregulation of known target genes argues that additional factors were not functionally present. The reason for this can range from the use of differentiation stage inappropriate factors, to the underlying cell preparation lacking a true SC identity.

3.2 Results and Discussion

3.2.1 Overexpression of Egr2 enhances the myelination frequency in primary Schwann cells

As the key players in peripheral nerve regeneration, SCs have gained much attention for nerve regeneration cell therapies in both peripheral and central nervous system applications. Upon acute injuries to the peripheral nervous system, they become activated and transdifferentiate into a pro-regenerative phenotype that secretes neurotrophic factors, clear myelin debris through autophagocytosis and recruitment of macrophages, organize into guiding structures within existing channels of basal lamina to what are called bands of Büngner, and ultimately remyelinate the regenerating axons, again taking on a mature phenotype [53]. Out of these attributes, remyelination and trophic support for recruitment of immune cells and axon guidance are key for cell therapies. Remyelination may be compromised, and can be especially hard to obtain at high efficiency using SCs derived through lineage reprogramming [54-56]. One possible approach to obtain highly functional, remyelinating SCs, is to harness the transcription factors that control the cell-intrinsic process of myelination, as outlined previously in this chapter. As we do not know if the human cell preparations have all the additional components present required for the key transcription factors to take effect, we turned to a proof-of-concept study in primary rat SCs. In this system, the cells are known to be able to myelinate, and functionalizing effects from transcription factor overexpression may be seen as an increase in the fraction of cells partaking in myelination. Thus the system

allows us to study effects of overexpression in a context where arguably all other components are available or can be activated.

To test if overexpression of the core factors of the feed-forward cascade (*Sox10, Oct6, Brn2* and *Egr2*) could potentiate myelination, we isolated and purified primary rat SCs from postnatal day 5 pup sciatic nerves (Figure 3-1), transduced them with a nuclear GFP lentiviral vector plus vectors for one or all of the above four factors and seeded them onto purified sensory neurons isolated from embryonic day 14.5 rat dorsal root ganglia. Compared with cells transduced with the nuclear GFP vector alone, only the group co-transduced with *Egr2*, but none of the other combinations, showed an increased number of myelin fragments (Figure 3-2A and B). Closer examination however showed a relatively poor overlap of GFP^+ cells with the myelin segments (Figure 3-2B). This could be due to poor co-transduction, but could also indicate that viral transduction itself is detrimental to myelination, and that the effects were not directly related to the overexpression of *Egr2*. We therefore used cells transduced with lentiviruses carrying *Egr2* with C-terminal epitope tags (*Egr2-Myc-DDK*), which allowed us to directly compare cells expressing the transgene to naïve, non-transduced cells. This experiment confirmed that *Egr2*-overexpressing cells myelinate at a higher frequency than naïve, non-transduced cells (Figure 3-2C), while transduction with nuclear GFP had no observable effect over naïve cells (Figure 3-3). An EdU assay carried out two days after myelination had been induced by the addition of ascorbic acid (five days total into the co-culture period) showed a decreased mitotic activity in the *Egr2*-transduced cells (Figure 3-2D and E), in line with *in vivo* reports[22,57]. This also confirms that the enhanced myelination was indeed a true functionalization, and not a positive selection of the

transduced cells, which would have instead been related to an increase in proliferation of *Egr2*-transduced cells. Only limited cell death was observed for all groups over the period of the co-culture, and a TUNEL assay carried out over the same time of co-culture confirmed a very low and comparable level of cell death between naïve and *Egr2*-transduced cells (Figure 3-2F). Thus, cell death through ER stress was not greatly manifested in our model of *Egr2* overexpression. Taken together, these results show that overexpression of *Egr2* can enhance the *in vitro* myelination frequency of primary SCs.

3.2.2 Egr2 overexpression does not induce upregulation of known target genes in human pluripotent stem cell-derived SC precursors under configurations tested

Encouraged by the *in vitro* results from primary rat SCs, we tested if *Egr2* overexpression could promote myelination in SC precursor-like cells derived from the iPS cell line BC1[58,59]. The cells were transduced with lentiviruses carrying a bi-cistronic expression vector for *mCherry* and *Egr2* (hereafter referred to as *mCherry-Egr2*), and a control transfection with *mCherry* alone at the same viral titer was carried out in parallel. Three days after transduction, most of the cells in the *mCherry-Egr2* group were dead, and an additional three days after this all the cells in this group were dead, while cells receiving the *mCherry* control vector were alive and robustly transduced (Figure 3-4). Titration the viral dose did not render viable transduced cells (data not shown). Given that the control transduction did not result in cell death, these results suggest that the overexpression of *Egr2* is responsible it.

Previous reports using primary murine SCs have observed an upregulation of *Egr2* with associated ER stress response upon treatment with retinoic acid both in monocultures and in co-cultures with sensory neurons. Interestingly, the study observed the induction of *CHOP* expression[60]. This is a transcription factor that induces expression of pro-apoptotic Bcl proteins. The effects of pro-apoptotic Bcl proteins can be mitigated by expression of anti-apoptotic Bcl proteins, such as *Bcl-xL*[61]. Overexpression of this factor in SCs has furthermore been able to prevent their apoptosis induced by high levels of glucose[62]. To test if overexpression of *Bcl-xL* could rescue the cells from the apoptosis induced by *Egr2* overexpression, and allow it to functionalize the cells, we cloned a multi-cistronic lentiviral expression vector containing *mCherry*, *Egr2,* and *Bcl-xL*. The constructs were confirmed on HEK 293T cells, that expressed *mCherry* upon transduction, and Western blot revealed the expression of the expected protein products (Figure 5). Transduction of hESC-derived SCPs did not induce apoptosis when *Bcl-xL* was co-expressed. Analysis of the mRNA expression profiles further showed a robust upregulation of *Egr2* transcripts, but none of previously identified target genes[63] showed any significant regulation (Figure 3-6). These results suggest that at least this preparation of ESC-derived SCs was not amenable to induction into a myelinating phenotype by *Egr2* overexpression.

3.3 Figures

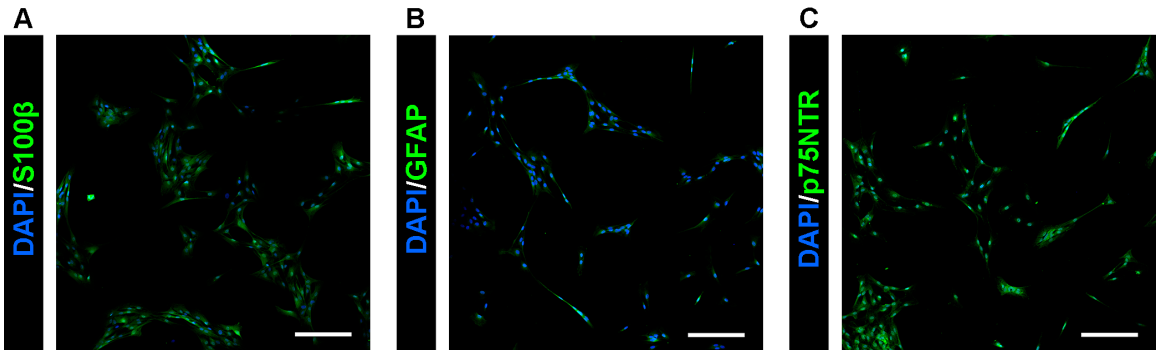

Figure 3-1 Isolated primary rat SCs are essentially pure and express canonical SC markers.

(A) S100β, (B) GFAP, and (C) $p75^{NTR}$ expression was confirmed in isolated SCs after purification. Scale bars = 200 μm.

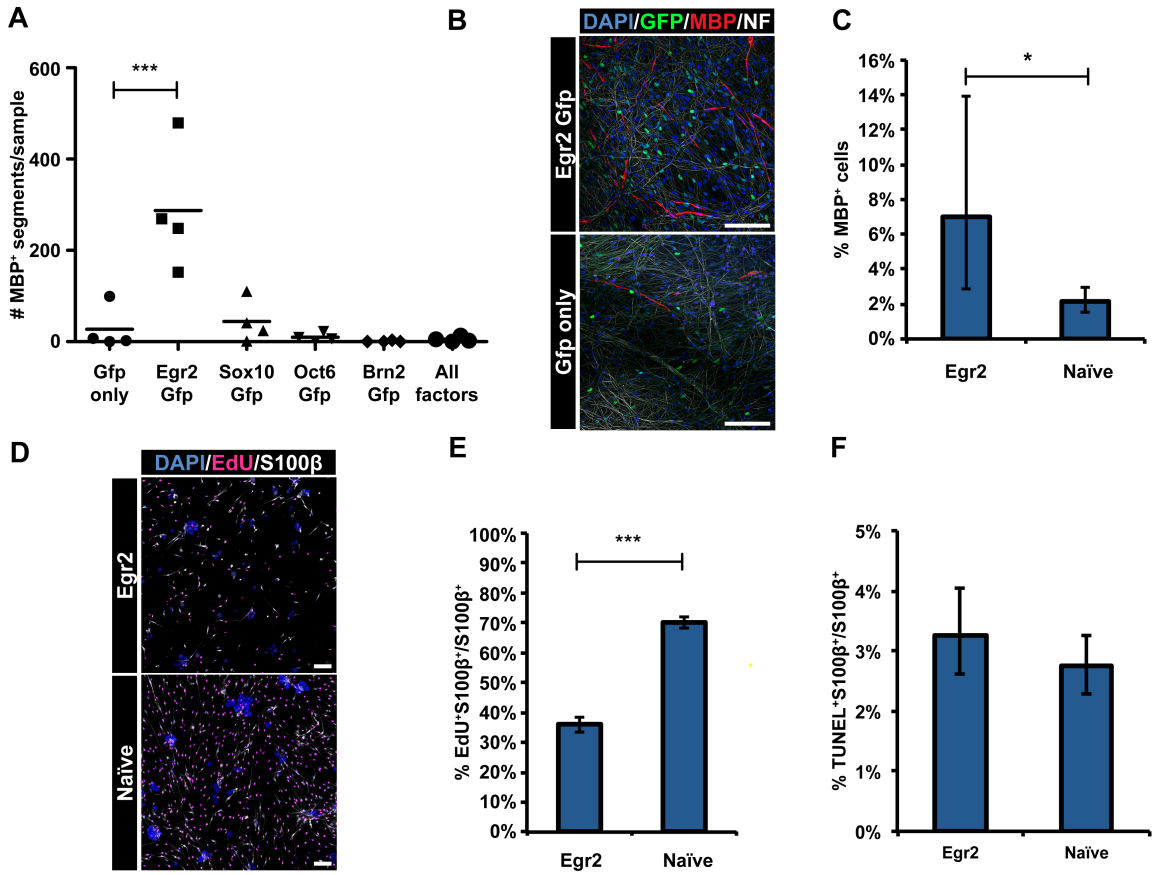

Figure 3-2 Enhanced myelination frequency through Egr2 transduction of rat Schwann cells in co-culture with rat DRG neurons.

(A-B) Increased number of myelin segments detected in *Egr2*-transduced group compared to *Gfp* only transduction, but not for the other factors *Sox10*, *Oct6* or *Brn2*. (C) Cells transduced with *Egr2-Myc-DDK* vector showed an increased myelination frequency compared to naïve, non-transduced cells. (D-E) Reduced proliferation seen by EdU assay over the first two days after addition of ascorbic acid to the co-culture seen in *Egr2*-transduced Schwann cells ($S100\beta^+$ fraction). (F) No significant difference in cell apoptosis between *Egr2*-transduced and naïve Schwann cells detected through TUNEL assay at day 2 after addition of ascorbic acid to co-cultures ($S100\beta^+$ fraction). Scale bars = 100 μm. * $P<0.05$. *** $P<0.0001$. Error bars = 95% CI.

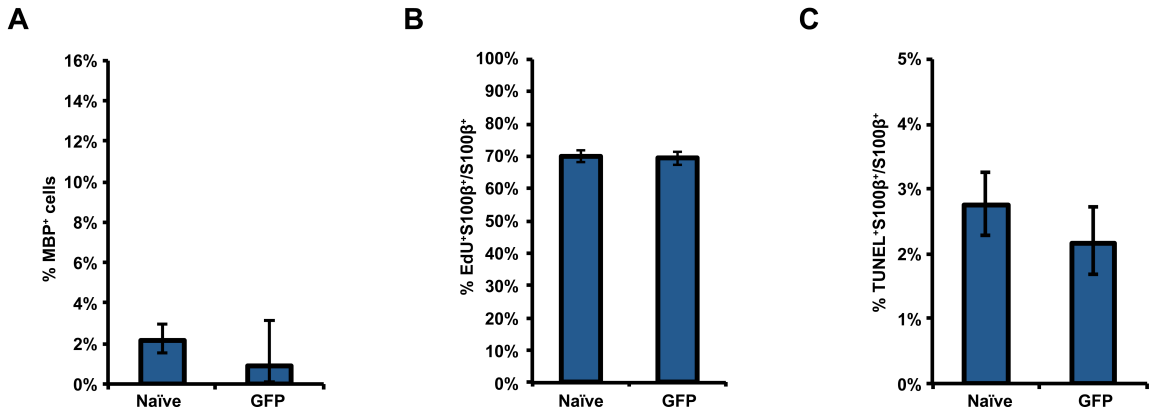

Figure 3-3 Lentiviral transduction with a GFP control vector does not affect SCs in co-culture with DRG neurons.

No difference was observed between GFP-transduced and naïve cells in terms of (A) myelination frequency, (B) proliferation ($S100\beta^+$ fraction) or (C) apoptosis ($S100\beta^+$ fraction) as evaluated at the same time points as those presented in Figure 2. Error bars = 95% CI.

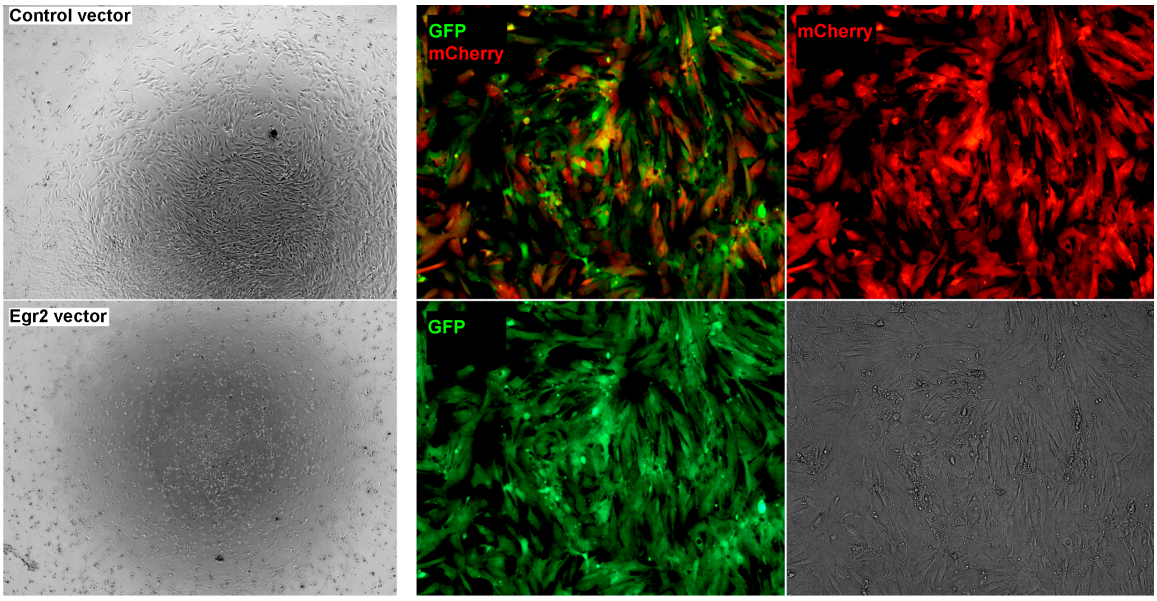

Figure 3-4 Overexpression of *Egr2* induces apoptosis in human pluripotent stem cell-derived SC precursors.

Left: *Egr2* overexpression causes widespread cell death in BC1-cell derived Schwann cell precursor like cells, while cells transduced with a control vector survived (day 6). Right: Almost all control cells expressed the transgene (mCherry), indicating that survival was not due to lack of transduction.

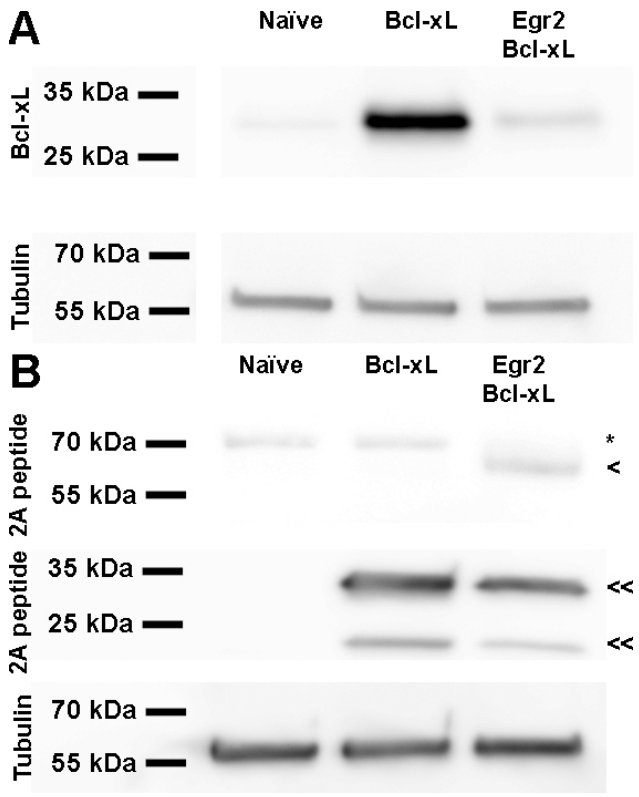

Figure 3-5 Confirmation of correct protein products in *mCherry, Egr2* and *Bcl-xL* multicistronic expression vectors.

Western blot analysis of protein lysates from HEK 293T cells either as naïve cells, transduced to overexpress *mCherry-Bcl-xL*, or *mCherry-Egr2-Bcl-xL*. (A) Probing for Bcl-xL revealed expression of the transgene in the expected samples. (B) Probing with anti-2A peptide revealed the expected products for (<) *Egr2*, and (<<)*mCherry*. * indicates a non-specific band detected above 70 kDa. Probing against tubulin was used as a loading control in both cases. Viral dose was normalized in (B) but not in (A), explaining the higher Bcl-xL expression observed for *mCherry-Bcl-xL* construct. These blots demonstrate that the viruses are functional and that the proteins in the polycistronic vectors are properly separated.

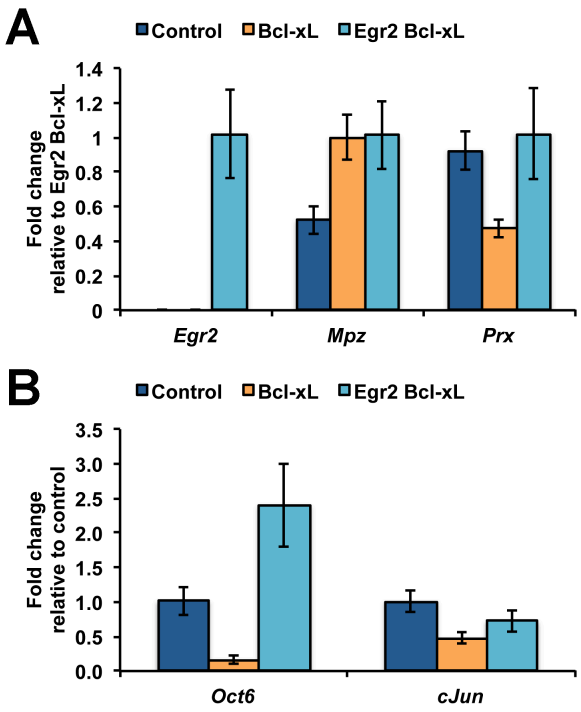

Figure 3-6 Overexpression of *Egr2* does not regulate known target transcripts in human pluripotent stem cell-derived SC precursors.

qRT-PCR results for transcripts known to be (A) positively regulated and (B) negatively regulated by *Egr2*, as analyzed at three days post-transduction with either control (*mCherry*), *mCherry-Bcl-xL*, or *mCherry-Egr2-Bcl-xL* lentiviral vectors, upon confirmation of reporter gene expression. *Atp5b* and *Ube4a* were used as reference genes upon determination of the most stable combination using the geNorm algorithm. n = 3 per group with two technical replicates per sample. Error bars = one standard deviation.

3.4 Tables

Antigen	Vendor	Catalog number	Dilution or Concentration	Host species and isotype
Myelin basic protein	BioLegend	808401	1:1000	Mouse IgG2b
Neurofilamen H	Millipore	AB1989	1:400	Rabbit IgG
S100β	DAKO	Z0311	1:400	Rabbit IgG
c-Myc	DSHB	AB_2266850	1:200 from concentrate	Mouse IgG1κ
p75 NGF Receptor	Sigma-Aldrich	N3908	1:1000	Rabbit IgG
Glial fibrillary acidic protein	DAKO	Z0334	1:500	Rabbit IgG
β (III) tubulin	Synaptic Systems	302304	1:1000	Guinea Pig IgG

Table 3-1 Antibodies used for immunocytochemistry

3.5 Materials and Methods

3.5.1 Cloning, production and titration of lentiviral vectors

Gateway entry clones for human *Sox10* (NM_006941.3), *Brn2* (BC051699.1), and *Egr2* (NM_000399.2) were obtained from the Johns Hopkins University High Throughput Biology Center. Human *Oct6* (NM_002699.3) was amplified by PCR from a TrueClone cDNA vector (Origene, plasmid SC317737, Rockville, MD, USA) using Q5 High-Fidelity Polymerase with the forward primer (5'- GCG AAT TCG GCG GCA TG -3') and reverse primer (5'- CAA TCT AGA TCA CTG CAC TGA GCC GG -3'), and cloned into pENTR1A no ccDB (w48-1), a gift from Eric Campeau (Addgene plasmid #17398, Cambridge, MA, USA), between the EcoRI and XbaI sites using the Rapid DNA Ligation Kit (Roche, Basel, Switzerland). Myc-DDK (DDK is also known as a FLAG tag) entry clones for *Egr2* were created by Gibson assembly with the PCR product from an *Egr2-Myc-DDK* containing plasmid (Origene RC212183, NM_000399.2) using forward primer (5'- CTG GAT CCG GTA CCG ATC GCC ATG ATG ACC GCC -3') and reverse primer (5'- TCG AGT GCG GCC GCG TTA AAC CTT ATC GTC GTC ATC CTT G -3') as insert and pENTR1A no ccDB (w48-1) digested with EcoRI-HF (NEB, Ipswich, MA, USA) as the backbone, and assembled using the Gibson Assembly Master Mix (NEB). Transfer vectors were then created through an LR clonase II reaction (ThermoFisher) into pLenti CMV Blast DEST (706-1), a gift from Eric Campeau (Addgene plasmid # 17451). A bi-cistronic transfer vector for *Egr2* and *mCherry* using a 2A peptide was created by cloning *Egr2* from the above transfer vector by PCR using forward primer (5'- TAT ATC TAG AAT GAT GAC CGC CAA GGC CGT AG -3') and

reverse primer (5'- TAT AGG ATC CCT ATC AAG GTG TCC GGG TCC GAG -3'), and ligating between the XbaI and BamHI sites of pUltra-hot, a gift from Malcolm Moore (Addgene plasmid #24130). A GFP with a nuclear localization signal (nuclear GFP) in the pLenti-CMV Blast DEST backbone was a gift from Donald Zack. All constructs had the CDS and insertion sites verified by Sanger DNA sequencing (Genewiz, South Plainfield, NJ, USA), and were expanded in Stbl3 cell hosts (Thermo Fisher Scientific, Lafayette, CO, USA).

For lentivirus production, HEK 293T cells, a gift from Donald Zack, were plated at 4 million live cells per plate in 8 ml of DMEM/F12 supplemented with 10% HI-FBS, 1x MEM non-essential amino acid solution, 1 mM sodium puryvate, penicillin (100 U/ml) and streptomycin (100 μg/ml, all from Thermo Fisher), onto poly-(D)-lysine-coated 10 cm dishes (Sigma-Aldrich, St. Louis, MI, USA). The next day, 15 μg/plate of the VSV-G envelope plasmid pMD2.g (Addgene plasmid #12259), 6 μg/plate of pMDLg/pRRE (Addgene plasmid #12251), 6 μg/plate pRSV-Rev (Addgene plasmid #12253), all gifts from Didier Trono, as well as 15 μg/plate of respective transfer vector was combined to a final plasmid concentration of 100 μg/ml. Then, linear poly(ethyleneimine) (Polymer Chemistry Innovations, Inc., Vista, CA, USA) was added to the mixture at a nitrogen to phosphate molar ratio of 6, and the polyplexes added to the cells for 4 hours before the media was exchanged. The following day, 10 mM sodium butyrate (Sigma-Aldrich) was added to the media. Two days after transfection, the supernatant was collected and viruses concentrated using Lenti-X concentrator (Clonetech, Palo Alto, CA, USA) according to the manufacturer's instructions, aliquots prepared in PBS and stored at -80^{o}C.

To determine the multiplicity of infection (MOI), purified primary rat SCs were infected with a serial 1:10 dilution of the nuclear GFP lentivirus in the presence of 8 μg/ml of polybrene (Sigma-Aldrich) over night, then detached four days after transduction using TrypLE Express (Thermo Fisher) and fixed with 1% paraformaldehyde in PBS before analyzing transduction efficiency by flow cytometry. Cells were considered positive when their expression level was above 99.9% of the cells in a non-transduced control sample. MOI was calculated for samples with a 2 – 20% transduction efficiency. After this, titration was performed using a PCR kit (Applied Biological Materials, Richmond, BC, Canada) on all other constructs and batches of lentiviruses, and their MOI determined by their relative viral titer to the nuclear GFP vector.

3.5.2 Western blot

HEK 293T cells were transduced with lentiviral vectors in the presence of 8 μg/ml polybrene (Sigma-Aldrich) over night. Once the cells displayed expression of the fluorescent reporter, they were washed with PBS, lysed on ice with 1x RIPA buffer (Cell Signaling Technology, Beverly, MA, USA), and 1x protease inhibitor cocktail (Sigma-Aldrich) in PBS for 5 minutes. The plates were scraped using a rubber policeman, and the lysate transferred to a pre-chilled tube. Following a brief sonication, samples were centrifuged at 14 000 xg for 10 minutes at 4^{o}C. The supernatant was collected, aliquots prepared and frozen down at -80^{o}C until further analysis. The protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL, USA) according to manufacturer's protocol.

Samples were thawed on ice and dissolved in 1x Laemmli's buffer with loading dye (Bio-Rad, Richmond, CA) and an additional 5% β-mercapto ethanol (Sigma-Aldrich) in PBS and samples denatured for 5-10 min at 95^{o}C. 20 μg protein was loaded per lane into 4-15% TGX gels (Bio-Rad) in 1x TGS buffer. PageRuler Plus pre-stained ladder (Thermo Fisher) was used for molecular weight determination. Gel was run at 30 V loading voltage followed by 200 V running voltage until loading dye reached the bottom of the gel. Gels were transferred to PVDF membranes using TransBlot Turbo systems and protocols (Bio-Rad), before rinsing in TBS with 0.1 % Tween 20 (TBST).

Blots were blocked with 5% non-fat dry milk (Bio-Rad) in TBST for 1 hour, before incubating with rabbit primary antibodies against 2A peptide (ABS31, 1:10000 dilution, Millipore, Billerica, MA, USA), or Bcl-xL (catalogue number 2764, 1:1000 dilution, Cell Signaling Technology) in 5% non-fat dry milk in TBST over night. Blots were then rinsed 5 times briefly and washed 3 times over 10 minutes with TBST before incubating with HRP-conjugated donkey anti-rabbit antibody (A16035, 1:10000 dilution, Thermo Fisher) for 1 hour at room temperature in above blocking buffer before washing as above.

Blots were developed using ECL reagent (GE Healthcare, Waukesha, WI, USA) for 1 minute and imaged using LAS 4000 system (GE Healthcare).

HRP was then deactivated by incubation with 30% hydrogen peroxide (Sigma-Aldrich) for 20 min at 37^{o}C, and inactivation confirmed using ECL development as above.

Blots were then probed with a loading control, mouse anti-tubulin (Sigma-Aldrich) as above, with secondary HRP-conjugated goat anti mouse (A16017, 1:10000 dilution, Thermo Fisher) with incubations and development done the same as above.

3.5.3 Cell isolation and purification

Primary rat SCs were isolated from Sprague Dawley rat postnatal day 4 pups using the methods described by Brockes [64] with slight modifications. Briefly, sciatic nerves were dissected and epineurium removed. The nerves were then digested with collagenase type I (Thermo Fisher) at 3 mg/ml in L-15 medium for 45 minutes at 37^{o}C, further treated with 0.375 mg/ml DNase (Thermo Fisher) and 0.0625% Trypsin EDTA (Thermo Fisher, diluted from 0.25% solution) for 15 min at 37^{o}C. The reaction was stopped by addition of DMEM containing 10% HI-FBS, nerves washed with L-15, and then titurated to release the cells and plated onto TCPS coated with collagen (Nutragen, 1:30 dilution, Advanced BioMatrix, Poway, CA, USA) in DMEM with high glucose, no sodium puryvate (Thermo Fisher), 10 μg/ml of piperacillin and ciprofloxacin (Sigma-Aldrich) and 10% HI-FBS (Thermo Fisher) (media composition hereafter referred to as D10). The day after plating, Ara-c was added to the media at 10 μM for 2-3 days, after which cells were cultured in D10 with 200 ug/ml of bovine pituitary extract and 2 μM forskolin (both from Sigma-Aldrich). Cells were passaged by detaching with Accutase (SigmaAldrich), incubated with anti-Thy1 from hybridoma supernatant at a 1:100 dilution (TIB-103, American Type Culture Collection, Manassas, VA, USA) for 30 minutes at 37^{o}C, washed with DMEM and incubated with rabbit complement diluted 1:8 (MP Biomedicals, Aurora, OH, USA), before plating at a 1:2 – 1:3 seeding ratio. Media was exchanged three times per week.

Mice with eGFP expression under the proteolipid protein promoter were gifts from Jeff Rothstein, originally derived in the lab of Wendy Macklin [65]. Primary mouse SCs were isolated in the same way from the sciatic, median and ulnar nerves of P4 Plp-eGFP pups, except that the epineurium was not removed and no Ara-C or anti-Thy1 treatment was performed. Transgene expression was confirmed in biopsies by epifluorescent microscopy prior to cell isolation, and only positive pups used. Nerves from the positive pups of one litter were pooled prior to digestion.

Sensory neurons were isolated from Sprague Dawley rat day 14.5 embryo dorsal root ganglia (DRG). After dissection of the DRGs, they were transferred into L-15 media to which 100 µl of 10 mg/ml collagenase type I, 40 µl of 0.25% Trypsin-EDTA, and 15 µl of 25 mg/ml DNase was added. The tissue was incubated at 37^{o}C for 45 min, washed twice and then plated onto 12 mm PDL/laminin-coated glass slides (Thermo Fisher) at 30 000 cells in a 100 µl droplet of Neurobasal media supplemented with 1x B27 supplement, 1x Glutamax (Thermo Fisher), 50 ng/ml NGF (Alomone Labs, Jerusalem, Israel), and 10 µg/ml of piperacillin and ciprofloxacin (Sigma-Aldrich). After 1.5 hours, media was added to fill the well. Cells were purified the next day by adding 10 µM of both uridine and 5-fluorodexuridine (FuDR, Sigma-Aldrich) for 2-3 days, kept 2 days in regular culture media, followed by another 2 day purification treatment. After this, mitotic cells could no more be observed in the cultures. Media was exchanged three times per week, and the cells underwent at least two media changes after completing the purification before initiating co-culture experiments.

3.5.4 Schwann cell and DRG neuron co-cultures

6 well plates of TCPS were coated with collagen (Nutragen, 1:30 dilution, Advanced BioMatrix), and purified rat SCs at passage 4 – 6 after isolation were seeded at 100 000 cells/well. The following day, 8 μg/ml of polybrene (Sigma-Aldrich) was added to the media together with the lentiviral vectors used for the respective assay (see below). The next day, the media was removed, cells washed two times with PBS, and then detached using Accutase (Sigma-Aldrich). The cells from one transduced well were seeded onto four cover slips with purified DRG neurons in media composed of EMEM (Quality Biological), 4 mg/ml D-glucose (Sigma-Aldrich), 50 ng/ml NGF, and 1% HI-FBS. After three days of co-culture, half the media volume was replaces and ascorbic acid supplemented to a final concentration of 50 μg/ml to induce myelination. Approximately half the media was replaced daily throughout the time of the co-culture, adjusting for some evaporation, with the ascorbic acid prepared fresh every time from powder. The half media change was done not to expose the cells directly to the ambient air, but to keep them constantly hydrated.

For comparing the effects of the different transcription factors, the cells received the vectors at an MOI of 5 for each vector used, except for *Sox10*, which was given at an MOI of 1.17 due to poor viral titers. Groups included nuclear *Gfp* vector alone, or the nuclear *Gfp* vector plus one or all of the transcription factors as pooled viral solutions. Samples were collected at three weeks after the first ascorbic acid addition.

To further investigate the effects of *Egr2*, cells were treated as above with lentiviruses encoding nuclear *Gfp* or *Egr2-Myc-DDK* (*i.e.* not combining the vectors) at

an MOI of 1, and compared to naïve, non-transduced cells. Samples were collected at three weeks after the first addition of ascorbic acid. *Egr2*-transduced cells were identified by the presence of the *Myc* epitope tag. This treatment was also used for the EdU and TUNEL assays, but at shorter time points (see below).

3.5.5 EdU incorporation assay

SC and DRG co-cultures were pulse-labeled by replacing half the media volume with fresh media containing 20 μM 5-ethynyl-2-deoxyuridine during the first and second media change at which ascorbic acid was also added. This was only done to samples used for proliferation assays: SCs transduced with the *Egr2-Myc-DDK* vector, *nuclear-GFP,* and naïve cells. Two days after the first addition, cells were fixed using 4% paraformaldehyde in PBS for 10 minutes at room temperature, and stored in PBS at 4^{o}C until staining. The cells were then labeled using the Click-iT® 555-EdU assay (Thermo Fisher), before staining for S100β with an Alexa Fluor 647 secondary as described below. Four samples per group were used, imaging two regions per sample and counting only S100β-labeled cells to specifically detect SCs. Samples were also stained with the anti-Myc antibody with an Alexa Fluor 488 secondary, but the anti-Myc had gone bad and this channel was excluded from the analysis.

3.5.6 TUNEL assay

SC and DRG co-cultures, for SCs transduced with the *Egr2-Myc-DDK* vector, *nuclear-GFP,* and naïve cells, as well as controls containing only DRG cells, were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, before immediately staining with the ApopTag Red kit (Millipore) according to the manufacturer's

instructions. Negative control samples using isolated DRG cells were prepared by omitting the TdT enzyme step. After this stain, the samples were stained as described for the EdU assay, again not using the anti-Myc stain in the analysis.

3.5.7 Immunocytochemistry

At the completion of the assays, samples were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and either directly processed or stored at 4^{o}C in PBS until staining. Samples were then permeabilized using 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 20 min at room temperature, rinsed three times with PBS, and then blocked for one hour in PBS containing 5% normal goat serum (Sigma-Aldrich). Primary antibody incubations were carried out over night at 4^{o}C in the blocking buffer, with antibody dilutions according to Table 3-1. Samples were then washed five times over 25 min with PBS, and incubated with secondary antibodies for one hour at room temperature. Apart from a Cy3-conjugated goat anti-mouse IgG2b antibody (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) used at a 1:200 dilution, all other secondaries were Alexa Fluor conjugated and raised in goat used at a 1:500 dilution (Thermo Fisher) in blocking buffer. The incubation was followed by five washes over 25 min with PBS, and samples were then mounted using ProLong Diamond Anti-Fade with DAPI (Thermo Fisher). Imaging was performed using a Zeiss LSM 780 system, and images presented are maximum intensity projections from confocal Z-stacks.

3.5.8 Statistical analysis

Statistical analysis was carried out using R (R Core Team 2015), and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Effects of the different transcription

factors were compared to the *Gfp* only control group (Figure 3-2A) using Dunnett's multiple comparison test. Direct comparisons of myelination frequency, EdU incorporation and TUNEL assays between groups were performed using Fisher's exact test, and 95% confidence intervals for the individual means, as depicted in the graphs, were computed using a binomial test. Results were considered statistically significant for $P<0.05$.

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CHAPTER 4 - USE OF OLIGODENDROCYTE PROGENITOR CELLS IN PERIPHERAL NERVE REGENERATION CELL THERAPIES

4.1 Introduction

Compared to the central nervous system (CNS), the peripheral nervous system (PNS) has a stronger inherent capability to regenerate. This is in large accredited to the myelinating glia cells in the PNS, the Schwann cells (SCs), which upon injury transdifferentiate into an activated phenotype that assists in myelin debris clearance, trophic support and axonal guidance, and eventual remyelination of the regenerating axons[1]. For the treatment of PNS injuries, much effort has been put forth to do away with the current gold standard for management of large nerve injuries, the autograft, which implies that suitable nerve tissue deemed to be less important has to be sacrificed and is associated with complications such as donor site morbidity and a shortage of suitable donor tissue[2]. The realization that endogenous SCs have a limited ability to populate large acellular grafts due to eventual senescence[3], and that primary autologous SCs are as hard to come by as the initial donor tissue itself, has led to much effort to derive SC analogs from stem cell sources to be used in cell therapies in conjunction with decellularized or synthetic nerve guides[4]. As opposed to other cells explored, *e.g.* neural or mesenchymal stem cells that may provide trophic support[5,6], SCs are unique from the perspective that they are the cells of the PNS capable of regenerative remyelination.

In the CNS, oligodendrocytes myelinate axons, and upon injury the oligodendrocyte precursor cells (OPCs) become activated to produce oligodendrocytes capable of remyelinating axons[7-12]. While progress is being made in cell preparation techniques for both SCs[13-22] and OPCs[23-30], only protocols for human pluripotent stem cell-derived OPCs have received FDA clearance for phase I clinical trials[31].

To date and to the best of our knowledge, there has not been any study investigating the feasibility of using oligodendrocytes for PNS therapies. We therefore set out to test if the PNS microenvironment can support primary OPCs, and if so, if the cells can serve a function in regenerative myelination and cell therapies for PNS injuries. Heterotopic engraftment of mouse OPCs isolated from perinatal brains showed a good survival in cryolesioned tibial nerves of non-obese diabetic (NOD)-severe combined immunodeficiency (SCID) mice, with multiple cells engaged in myelination one month after engraftment. Interestingly, the myelinating cells were predominantly found to be of a SC-like phenotype, suggesting that the microenvironment not only supports the survival of the cells and remyelination of the axons, but also that the cells undertake a phenotype appropriate to the PNS, expressing periaxin, a component of PNS but not CNS myelin[32], the SC marker S100β, and forming a one-to-one relation closely approximated to the axons being myelinated. The engrafted cells further lacked expression of oligodendrocyte lineage markers Olig2 and myelinating oligodendrocyte glycoprotein (MOG). Our results are thus the first to demonstrate that cells of the oligodendroglia lineage can be effectively used in peripheral nerve cell therapies, and that the PNS microenvironment promotes their differentiation into a SC-like phenotype.

4.2 Results

4.2.1 OPCs engraft and remyelinate in the PNS

To test whether or not OPCs can engraft and remyelinate in the PNS microenvironment, we performed heterotopic engraftment of acutely isolated OPCs from postnatal day 4 – 5 mouse pups brains expressing an eGFP reporter under the proteolipid protein promoter (Plp-eGFP mice)[33], and purified using magnetic activated cell sorting against the O4 antigen, into cryolesioned tibial nerves of NOD/SCID mice. Cryolesioning, or cryoanalgesia, was used as a way to kill the host cells *in situ*[34] and reduce competitive remyelination from the host SCs while preserving some of the native nerve architecture and basal lamina tracts[35-38] This is a method used clinically for long-term pain management[38,39]. The cell preparations were predominantly of an immature oligodendrocyte phenotype, with the majority of the cells expressing O4, A2B5 and Olig2 (Figure 4-1). One month post engraftment, numerous $eGFP^+$ cells were found distributed throughout the lesion site (Figure 4-2A). As the eGFP expression indicated Plp promoter activity, a major component of myelin, it indicated not only that the cells had survived, but also that these cells had obtained a more mature phenotype. Staining against myelin basic protein and neurofilament revealed numerous cells engaged in myelination of the regenerating axons (Figure 4-2B-C).

4.2.2 OPCs engrafted into the PNS transdifferentiate into myelinating SC-like cells

The observation that myelinating OPCs were found to closely associate with their axons (Figure 4-2B) made us ask whether or not they had undertaken a SC-like phenotype. SCs namely undergo a process known as radial sorting, where the pro-myelinating SCs associate with large caliber axons to be remyelinated in a one-to-one relation, as opposed to oligodendrocytes which myelinate multiple axons[40]. Numerous observations have shown myelination of CNS axons by SCs in certain focal injury models[41-43] as well as in multiple sclerosis patients[44,45], and recent lineage tracing experiments established that these cells are derived from local OPCs[43]. To determine if the myelinating cells were of a SC phenotype, we co-stained for S100β and MBP. 64 % of the MBP^+ cells were also S100β positive (Figure 4-3A-C), indicating that they were of a SC-like phenotype. While astrocytes also express S100β, and OPCs can differentiate into astrocytes[46], an astrocyte fate in these cells can be excluded based upon their engagement in myelination. To further confirm their SC-like identity, we quantified the percent of ensheathing graft-derived cells that were positive for periaxin. 93% of graft-derived cells associated with axons were found positive for periaxin (Figure 4-3D-E). We further examined the expression of myelinating oligodendrocyte glycoprotein (MOG) to understand if the myelinating or pro-myelinating cells would express a marker for CNS myelin. Only one out of the 187 analyzed Plp-$eGFP^+$ cells were found positive for MOG, while control stains of adult mouse corpus callosum were clearly positive (data not shown). None of the cells expressing the Plp-eGFP reporter engrafted into the cryolesioned tibial nerves were found to express the oligodendrocyte lineage marker

Olig2 (0 out of 597 cells analyzed), while it was readily detected in $eGFP^+$ cells in the cortex of adult Plp-eGFP mice (data not shown). Collectively, these results argue that the myelinating graft-derived cells are predominantly of a SC-like identity.

4.3 Discussion

To the best of our knowledge, this constitutes the first report of OPCs capable of engrafting and remyelinating axons in a peripheral nerve injury model. With recent advances in lineage reprogramming techniques for OPCs[23-30], our findings highlight them as new candidates for cell therapies to treat peripheral nerve injuries. This is a highly relevant proof-of-concept for cell therapies, as OPC preparation methods from human pluripotent stem cells have progressed to the point where they are evaluated in clinical trials, and have been shown capable of myelinating axons[31].

Our results indicate that the PNS microenvironment causes them to undertake a SC-like phenotype. With 93% of the cells engaging axons expressing the PNS myelin component periaxin suggests that the cells undertake a myelinating phenotype appropriate to the PNS with high efficiency. Out of the Plp-eGFP reporter and MBP double-positive cells, 64% also expressed the mature SC marker S100β. While this is the first report of the PNS microenvironment supporting survival and SC-like differentiation of OPC progeny, the appearance of SCs in the CNS has been known for a long time in models of focal demyelination[41-43] as well as in patients suffering from multiple sclerosis[44,45]. As the SC appeared at the center of the lesion site from where astrocytes had been ablated, but that oligodendrocyte myelination was seen in the periphery of the lesion site where astrocytes were once again present, it was believed that astrocytes

maintain a CNS to PNS glia boundary, restricting SC migration into the CNS[41]. Further evidence for the involvement of astrocytes in determining the eventual myelinating phenotype in CNS lesions came from OPC engraftment studies into focal demyelination of the spinal cord. When the population was purified from astrocytes and engrafted into ethidium bromide-induced demyelinated lesions, extensive SC myelination was observed. However, if the engrafted cells still contained astrocytes, oligodendrocyte myelination would predominate[47]. The idea that astrocytes act to restrict migration of SCs held true when the migration of SCs isolated from male donors were engrafted into lesions in female hosts, thereby possible to track by *in situ* hybridization against the Y-chromosome, where the engrafted SCs were contained within an astrocyte boundary. As pointed out by the authors, it is however not certain that the restriction demonstrated by the astrocytes at a lesion site like this necessarily translate into other astrocyte-containing areas of the CNS[48]. Another study using the same approach to track the progeny of male donor-derived PSA-NCAM$^+$ neural precursor cells[49], an early glial progenitor which *in vitro* gives rise to mostly astrocytes and oligodendrocytes when transferred to adherent substrates[50], found the Schwann cell myelination at the site to be graft-derived[49]. Subsequent studies have shown that engraftment of OPCs into demyelinated CNS lesions ablated from astrocytes also display SC myelination[51,52], and that the SCs observed in this setting are graft-derived[52]. The CNS however contains multiple cell-types that have been shown capable of undertaking a SC-like phenotype, from neuroepithelial stem cells present during embryonic development[53], to cells present in perinatal and adult tissue such as olfactory ensheathing cells[54,55], tanycytes, pituicytes[56], as well as SCs associated with autonomic nerve fibers of large blood vessels. It is therefore possible that the

primary isolates contain a small subpopulation of contaminating cells that account for the observed SC-like remyelination. This also holds true for our study, as these cells are known to express the cell surface marker O4 used to purify our cultures[54-56]. It is however the much more implausible interpretation of the data, as OPCs are a highly numerous population. Even in the adult brain, they constitute 4-5% of the total cell population[57,58]. To solidify whether or not the observed SC-like myelination could be OPC-derived was thus studied through lineage tracing experiments in focal demyelination models of the spinal cord without any cell engraftment, and found that the observed SC-like cells were predominantly, derived from locally recruited OPCs[43].

What is then the connection between astrocytes and their control over the OPC fate? *In vitro* experiments have shown that astrocyte monolayers secrete factors that prevent type-2 astrocyte differentiation of OPCs of the optic nerve[59]. This is, at least in part, believed to be through their secretion of Noggin, an inhibitor of BMP signaling[60-62]. As neuroepithelial stem cells could be induced to obtain PNS competency though BMP-2/4 treatment[53], and findings that OPCs and cortical precursors could be induced into multipotent stem cells by first exposing them to bFGF followed by BMP[63,64], led to the proposition that astrocyte depletion in ethidium bromide-induced focal lesions also ablated the cell source for Noggin and allowing for activation signaling pathways that enable SC differentiation[65]. It was later shown that X-ray irradiated ethidium bromide-induced focal demyelination lesions in the spinal cord indeed had BMP-2/4 expression but lacked Noggin, and that transfection of the OPCs with a Noggin expression vector prior to engraftment would prevent SC-like differentiation[52], much like previous studies co-engrafting astrocytes with the OPCs[47]. These studies thus argue that inhibition of

BMP signaling by astrocyte-derived Noggin maintains the normal fate commitment of OPCs into oligodendrocytes. In the case of peripheral nerve tissue, Noggin is virtually absent relative to the levels seen in the brain or spinal cord[66]. It is therefore plausible that the same mechanisms accounting for the transdifferentiation seen in CNS demyelination models are at play also upon heterotopic engraftment into the PNS, although we do not have direct evidence for this. Blocking BMP signaling, *e.g.* with Noggin, would have secondary effects on PNS regeneration as it would act directly on the regenerating axons and block their regrowth[67,68]. We deemed knockdown of specific BMP receptors in the engrafted cells as unfeasible and beyond the scope of this study, as multiple receptor combinations and signaling pathways may converge[69].

The lack of $Olig2^+$ cells in our study suggests that the progenitor pool has been largely depleted. This indicates that while the PNS microenvironment can maintain the differentiated progeny over the time of our study, long-term maintenance of undifferentiated OPCs is very limited. We see that this could be through two distinct mechanisms: direct terminal differentiation of the OPCs, or death of any self-renewing OPCs, leaving behind only the differentiated progeny. It should be stated that we isolated the cells from perinatal pups, where the predominant progeny of OPCs in the brain has been shown to be mature oligodendrocytes, they still maintain a sizeable fraction of 40% OPC progeny[70]. The extent to which environmental factors vs. a cell-intrinsic predisposition plays a part in this fate commitment is unknown. While it is true that we select for a more mature progeny through O4 expression than the NG2 cell studied by Zhu *et al.*[70], O4 cells isolated from perinatal brains can however be propagated *in vitro* in the presence of FGF2 and PDGF-AA[71], and results from *in vivo* studies show that the O4

antigen presence is not directly related to a terminal fate commitment, but that O4 cells continue to proliferate in the perinatal brain[72]. In the uninjured brains of mice, studies have shown compelling evidence for both direct fate commitment[73], as well as for asymmetric division[74]. In response to injury, OPCs become activated to proliferate and migrate to the lesion site. Injuries to the CNS trigger both differentiation and self-renewal of the OPC population[11,75-79], and repeated focal brain demyelination does not deplete the OPC pool[80], which would argue that the normal response maintains a population of progenitors. Focal injuries however rely largely on OPC recruitment from adjacent tissue[81], which is not present in our PNS model. Upon chronic demyelination through systemic cuprizone administration, continuous treatment depletes the progenitor cell pool. The progressive decline in OPC number suggests that the cells have a restricted capacity to recover under continuous pressure to proliferate[82]. Therefore, in addition to the possibility that OPCs cannot survive undifferentiated in the PNS over long periods due to lack of an appropriate niche, depletion of the population may also be due to extensive and direct differentiation and exhaustion due to a maintained pressure to proliferate.

We further observed a near complete absence of MOG expressing cells at the graft site, suggesting that either differentiation towards mature oligodendrocytes or their survival is highly limited in the PNS microenvironment. As we did observe one MOG^+ cell at one month post-engraftment, the cells appear to still have an ability to form oligodendrocytes, although the efficiency thereof may be limited. Previous studies in optic nerves suggest that oligodendrocyte survival depends upon axon-derived support[83,84]. This is in contrast to SCs, who become activated upon denervation to

proliferate and repair the injury site[1]. It is therefore likely that while the PNS microenvironment may not prevent oligodendrocyte differentiation of the OPCs, there is a selective advantage for the SC-like progeny explaining our observed results.

Finally, it appears that the niche plays a large role in OPC fate commitment, phenotype and function. All cases where SC-like differentiation of OPCs has been observed were associated with significant perturbations of their niche. This is an interesting concept also for CNS regeneration, as the employment of artificial niches may direct the differentiation of OPC progeny to facilitate their survival and function in regeneration. Two recent reports have shown axon regeneration in spinal cord injury models where hydrogels delivering growth factors were employed[85,86]. In one of the studies, the hydrogel was used to provide tropic cues to promote axonal extension through glial scar tissue. It is however possible that the growth factors altered the phenotype and function of the glial cells. Interestingly, it was reported that axons grew along reactive astrocytes in this setting (Figure 5G in Anderson *et al.*[85]), identified by the astrocyte marker GFAP. However, GFAP is also expressed in SCs, but becomes suppressed upon myelination[40]. It is tempting to speculate that the observed cell may have been a pre-myelinating SC associated with the axon, and that the growth factors delivered by the hydrogel had provided a niche in which OPCs (or other cells recruited from the surrounding tissue) had transdifferentiated into a phenotype supporting regeneration. Likewise, Lu *et al.*[86] used a fibrin gel carrying a cocktail of growth factors for the purpose of supporting the survival of the engrafted neural stem cells, but the impact of these factors and the artificial niche created by the hydrogel was not considered to act upon resident glia. The knowledge that OPC fate is controlled by its niche should

therefore be taken into consideration and investigated as an approach to promote regeneration also after spinal cord and brain injuries.

4.4 Figures

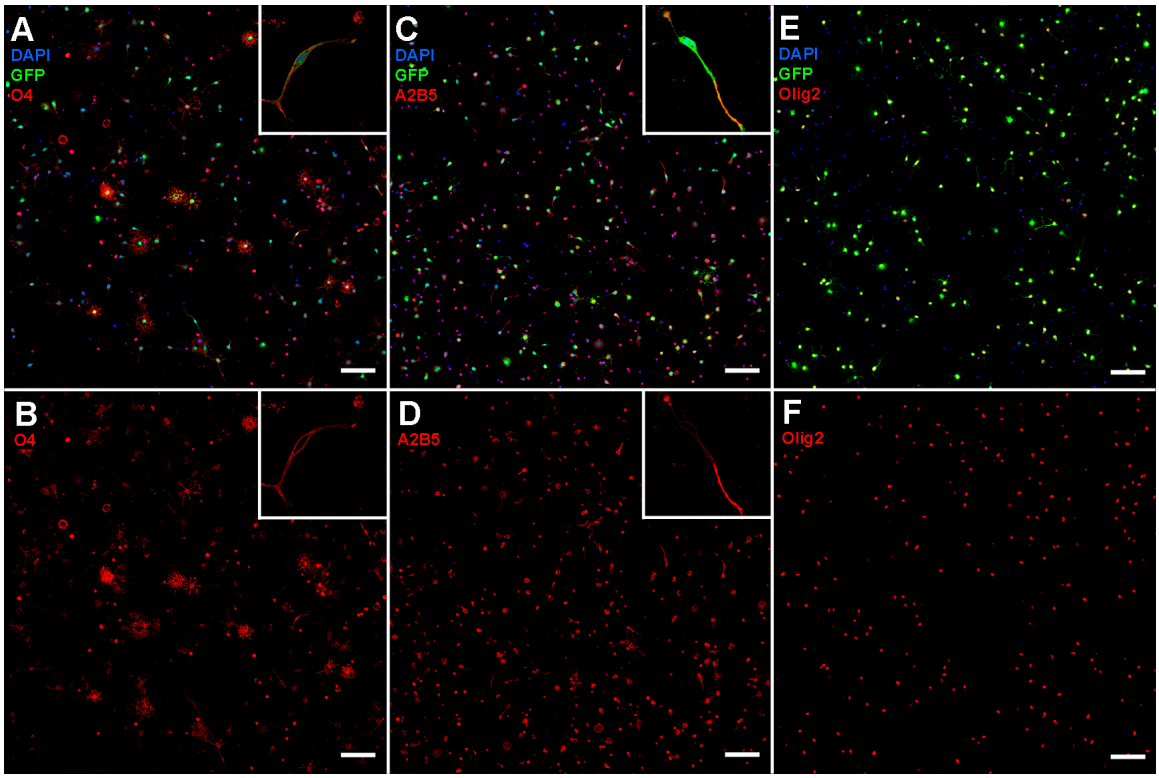

Figure 4-1 Expression of oligodendrocyte precursor cell markers was confirmed in isolated cells from P4-P5 mouse brains.

(A-B) O4 staining and cell membrane localization was confirmed in the isolated cells (C-D) A majority of the cells also expressed the cell-surface marker A2B5, and (E-F) were positive for the transcription factor Olig-2. Inserts in A-D show orthogonal projections of images at higher magnification. GFP expression is under a CAG promoter. Scale bars = 100 μm.

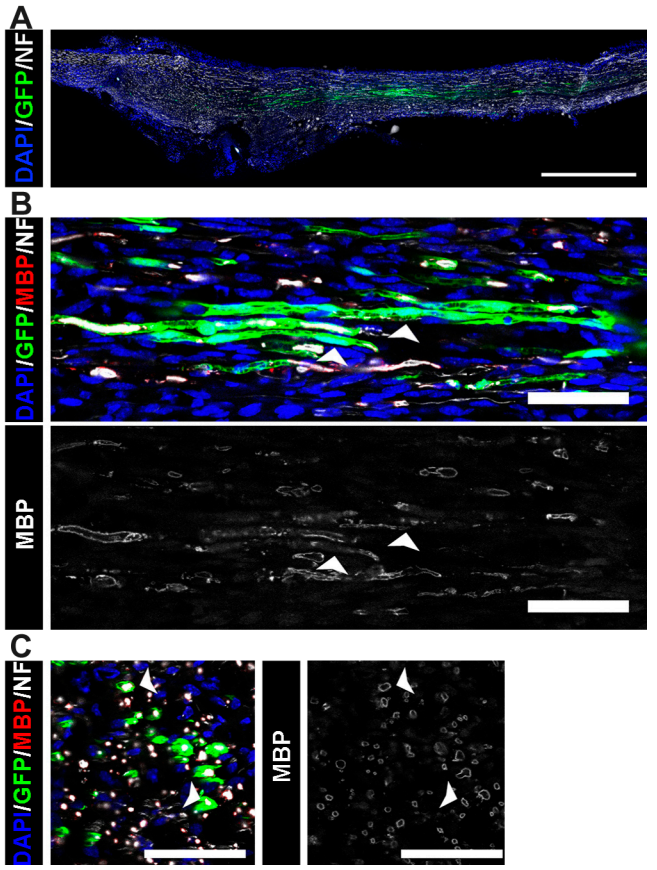

Figure 4-2 Engrafted OPCs survived and remyelinated after engraftment into cryolesioned tibial nerves of NOD/SCID mice.

(A) Plp-eGFP positive cells were found throughout the nerve, and (B-C) were found to engage in remyelination of regenerating axons one month after engraftment, as seen in (B) longitudinal and (C) transverse cross-sections. Arrowheads in (B) indicate graft-derived cells that are closely associated with the axons they myelinate. Arrowheads in (C) show examples of MBP^+ graft-derived cells. Scale bars are 1 mm in (A), and 50 μm in (B-C).

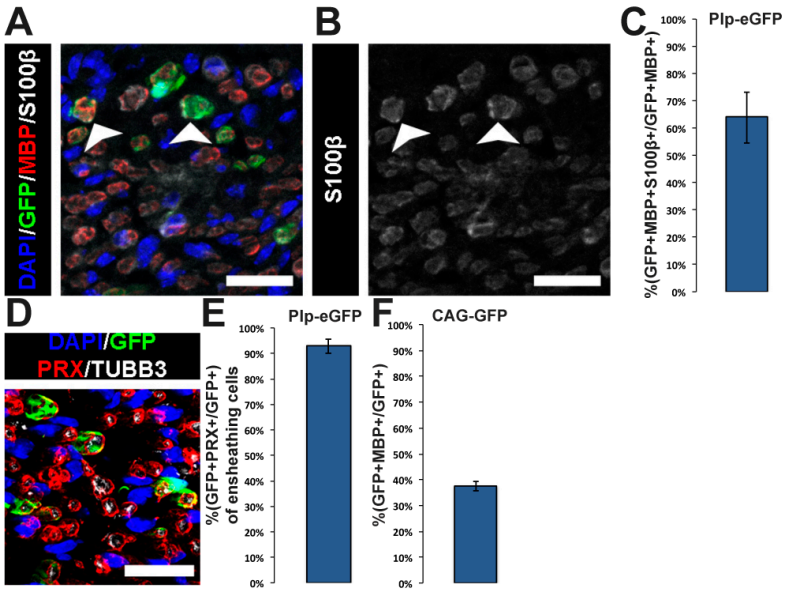

Figure 4-3 OPCs transdifferentiated into a SC-like phenotype upon engraftment into the cryolesioned tibial nerves of NOD/SCID mice.

(A-C) Co-expression of MBP and S100β in Plp-eGFP^{+} graft-derived cells. Arrowheads indicate graft-derived cells that were double-positive for S100β and MBP, indicating a SC-like phenotype. (C) 64% of the GFP^{+}MBP^{+} cells were also p S100β^{+}. (D-E) The majority of Plp-eGFP^{+} cells associated with axons expressed the peripheral myelin component periaxin. (E) 93% of axon-associated Plp-eGFP^{+} cells were found positive for periaxin. (F) 38% of the entire graft-derived population from CAG-GFP donors was found positive for MBP. Error bars in (C, E, F) = 95% confidence intervals. (C) n = 109 cells analyzed between 5 animals. (E) n = 368 cells analyzed between 4 animals. (F) n = 2790 cells analyzed between 6 animals. Donor animal for experiment indicated above Figures (C, E, F).

4.5 Tables

Antigen	Vendor	Catalog number	Dilution or Concentration	Host species and isotype
MBP	BioLegend	808401	1:1000	Mouse IgG2b
Neurofilamen H	Millipore	AB1989	1:400	Rabbit IgG
S100β	DAKO	Z0311	1:400	Rabbit IgG
O4	Sigma-Aldrich	O7139	1 µg/ml	Mouse IgM
A2B5	Sigma-Aldrich	8229	5 µg/ml	Mouse IgM
Olig2	Millipore	AB9610	1:500	Rabbit IgG
β (III) tubulin	Synaptic Systems	302304	1:1000	Guinea Pig IgG
MOG	Millipore	MAB5680	1:200	Mouse IgG1
Periaxin	Novus	NBP1-89589	1:100	Rabbit IgG

Table 4-1 Antibodies used for immunocytochemistry and immunohistochemistry

4.6 Materials and Methods

4.6.1 Cell isolation

Donor Plp-eGFP mice derived in Wendy Macklin's lab[33] were a kind gift from Jeffrey Rothstein. C57BL/6-Tg(CAG-EGFP)131Osv/LeySopJ (referred to as CAG-GFP) male and C57BL/6 wild-type females were obtained from Jackson Laboratories Inc. (Bar Harbor, ME, USA). At postnatal day 4-5, brains from pups positive for the reporter were dissected and the olfactory bulbs and spinal cord discarded. The tissue was dissociated using neural tissue disassociation kit (P) (Miltenyi Biotech, Auburn, CA, USA) and $O4^+$ cells purified using MACS anti-O4 beads and MS columns (Miltenyi Biotech) according to the manufacturer's instructions. A subset of the cells were plated onto PDL/laminin-coated glass cover slips (Corning BioCoat, Thermo Fisher, Waltham, MA, USA) at 40 000 cells in a 50 µl volume of Bottstein and Sato's serum-free medium (as described by Watkins *et al.*[87], omitting T3) supplemented with 10 ng/ul human recombinant PDGF-AA (PeproTech, Rocky Hill, NJ, USA). After 3-4 hours at 37°C, 5% CO_2, the cells had adhered and the wells were filled. Cells were fixed for purity assessment the following day using 4% paraformaldehyde in PBS for 15 min.

4.6.2 Immunocytochemistry

For intracellular antigens, the cells were permeabilized using 0.3% Triton X-100 (SigmaAldrich, St. Louis, MO, USA) in PBS for 20 min at room temperature. This was not done for surface antigens with extracellular epitopes. Samples were rinsed thee times with PBS prior to blocking for one hour at room temperature using 5% normal goat

serum (Sigma-Aldrich) in PBS. Primary antibodies were incubated over night at 4°C in blocking buffer. Samples were washed five times over 25 min with PBS before incubating with AlexaFluor-conjugated secondary antibodies (1:500 dilution, Thermo Fisher) for one hour at room temperature. Samples were once again washed 5 times over 25 min with PBS and mounted using ProLong Diamond anti-fade with DAPI (Thermo Fisher). Primary antibody omission controls were performed, and sampels imaged using an LSM 780 confocal microscope system (Carl Zeiss Microscopy GmbH, Jena, Germany).

4.6.3 Cryolesioning, cell engraftment and tissue processing

Cell suspensions of acutely isolated cells were prepared in HBSS w/o calcium or magnesium (Mediatech, Herndon, VA, USA) and placed on ice until engraftment. Adult male NOD.CB17-Prkdc^{scid}/J mice were obtained from Jackson Laboratories to serve as hosts. Mice under inhalation anesthesia (1-3% isoflurane) had the tibial nerves exposed and cryoanalgesia induced just distal to the bifurcation from the sciatic nerve by freezing it with a piece of dry ice. This was repeated three times with thawing in between before the nerve was transected just proximal to the cryolesioned site, 2 µl cell suspension containing 80 000 cells was injected into the center of the cryolesioned site using a Hamilton syringe, and direct nerve anastomosis was performed just proximal to the freeze/thaw site before the wound was closed using surgical clips. Animals were monitored for the one month duration of the experiment, displaying normal healing behavior without any adverse effects. After one month, the animals were sacrificed by transcardial perfusion, first with PBS and then with a 4% paraformaldehyde solution in PBS before the tissue was harvested for 2 hours of post-fixation in 4% paraformaldehyde

at 4°C. Nerves were transferred to 15% sucrose in PBS for 2-3 days at 4°C, followed by 30% sucrose for the same duration before embedding into Tissue-Tek O.C.T. (Electron Microscopy Sciences, Hatfield, PA, USA) and sectioning to 7 or 10 μm sections using a cryostat onto Superfrost Plus microscope slides (Thermo Fischer). Control samples were prepared from mouse brain and sciatic nerve.

4.6.4 Immunohistochemistry

Samples were permeabilized using 0.3% Triton X-100 (Sigma-Aldrich) in PBS, followed by 2 x 2 min washes with PBS before blocking with M.O.M. Mouse IgG Blocking Reagent (Vector Laboratories, Burlingame, CA, USA) for one hour. Samples were washed for 2 x 2 min with PBS, and incubated with M.O.M. Diluent (Vector Laboratories) for 5 minutes. All steps so far were performed at room temperature. Primary antibodies were then incubated in M.O.M. diluent over night at 4°C according to Table 4-1. The following day, samples were washed five times over 25 min with PBS, and secondary antibodies were incubated for one hour at room temperature, at 1:200 dilution for Cy3-conjugated anti-mouse IgG2b (Jackson Immuno Research Laberatories Inc., Avondale, PA), and 1:500 for all AlexaFluor-conjugated antibodies (Thermo Fisher). Samples were once again washed 5 times over 25 min with PBS, and then treated with Antifluorescence Eliminator Reagent (Millipore, Billerica, MA, USA) according to the manufacturer's protocol and mounted using ProLong Diamond Mounting Reagent with DAPI (Thermo Fisher) over night. Controls where primary antibodies were omitted were performed on known positive control samples to account for non-specific binding and cross-reactivity. For antigens normally not found in PNS tissue, mouse brain sections

served as positive controls. Samples were analyzed using a Zeiss LSM 780 scanning laser confocal microscope system (Carl Zeiss Microscopy GmbH).

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CURRICULUM VITAE

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Born February 22nd, 1984 in Pattijoki, Finland

EDUCATION

Ph.D. in Materials Science and Engineering, Johns Hopkins University, Baltimore, MD, USA, 2016

Supervisor: Dr. Hai-Quan Mao (Materials Science) Co-advisor: Dr. Ahmet Höke (Neuroscience)

- Johns Hopkins University Bootcamp for Bioentrepreneurs
- Preparing Future Faculty Certificate Program

M.Sc. in Engineering Biology, Devices and Materials in Medicine, Linköping University, Sweden, 2010

Supervisor: Dr. Joanne M Hackett, Examiner: Dr. Olle Stål

RESEARCH AND WORK EXPERIENCE

Graduate Research Assistant, Department of Materials Science and Engineering, Johns Hopkins University, 2011-2016

- Developed an in vitro transfection protocol using DMSO-treatment and L-PEI/DNA nanoparticles to enable >90% transfection efficiency in human immortalized Schwann cells.
- Enabled both serial and dose-dependent transfection of human pluripotent stem cell-derived Schwann cells through nanoparticle polymer optimization and synthesis of modified mRNA.
- Designed a hydrogel system to engraft and retain highly viable cells for peripheral and central nervous system cell therapies and regeneration, enabling vascularization of the graft.
- Screening of candidate transcription factors identified Egr2 overexpression to increase Schwann cell myelination frequency.

• Found oligodendrocyte progenitor fate commitment to be controlled by their niche, adapting a functional, myelinating Schwann cell-like phenotype when employed in cell therapies for peripheral nerves, and thus constituting a novel source of myelinating cells for peripheral nerve regeneration.

Research Assistant, Department of Clinical and Experimental Medicine, Linköping University 2010

Lab of Gunnar Kratz – Designed and developed an in vitro composite for skin cell culture models. Created highly porous nanostructured scaffolds to study substrate effects on fibroblast phenotype.

Lab of Per Aspenberg – Developed nanostructured tissue engineering scaffolds for bone and cartilage regeneration.

M.Sc. Thesis Research, Department of Clinical and Experimental Medicine, Linköping University 2010

Designed a porous nanofiber scaffold that would create a microenvironment promoting regeneration after spinal cord injuries by providing mechanical support, topographical guidance and selective cell and nutrient transport.

Salesman, TeliaSonera, Sweden 2004-2009 – Store salesman, providing telecommunication services and solutions to private and corporate customers. Received internal sales training.

Group Coordinator, LinTek, Sweden 2009 – Led a group of approximately fifty people catering to 40 visiting companies.

Head of Recruitment, Section of Engineering Biology, LiTH, Sweden 2007-2008

PUBLICATIONS

Articles

• Tammia, M., Mi, R., Choi, J., Shinn, D., Li, Y., Höke, A., Mao, H.Q. Oligodendrocyte precursors obtain a Schwann cell-like phenotype and remyelinate axons upon engraftment into peripheral nerves, *In preparation*

• Tammia, M., Mi, R., Zhu, A., Shinn, D., Höke, A., Mao, H.Q. Increase in myelination frequency through Egr2 overexpression in Schwann cells, *In preparation*

• Li, X., Tzeng, S., Liu, X., Tammia, M., Cheng, Y.H., Rolfe, A., Sun, D., Green, J., Wen, X., Mao, H.Q. Enhancing neuronal differentiation of human neural stem cells through nanoparticle-mediated neurogening-2 transfection for brain tissue regeneration *Biomaterials* **84**, 157-166, (2016).

• Khalifian, S., Sarhane, K.A., Tammia, M., Ibrahim, Z., Mao, H.Q., Cooney, D.S., Shores, J.T., Lee, W.P.A., Brandacher, G. Stem cell-based approaches to improve nerve regeneration in reconstructive transplantation, *Arch. Immunol. Ther. Exp.* **63**, 15-30, (2013).

• Krick, K., Tammia, M., Martin, R., Höke, A. & Mao, H.Q. Signaling cue presentation and cell delivery to promote nerve regeneration. *Current Opinion in Biotechnology* **22**, 741-746, (2011).

Textbook Chapter

• Tammia, M., Rakar, J. & Hackett, J. in *Advances in Medicine and Biology* Vol. 26 (ed Leon V. Berhardt) (Nova Science Publishers, Inc., 2011).

HONORS AND AWARDS

• Reviewer for the Journal of Tissue Engineering and Regenerative Medicine 2011-2015

• The Foundation Olle Engqvist Byggmästare 2010, 2012-2014
Fellowships towards Ph.D. studies at the Johns Hopkins University

• The Foundation BLANCEFLOR Boncompagni-Ludovisi, née Bildt 2011-2012
Fellowships towards Ph.D. studies at the Johns Hopkins University

• Hans Werthén fonden 2013
Fellowship towards Ph.D. studies at the Johns Hopkins University, 2014

• BSRT International Summer School on Innovative Approaches in Regenerative Medicine – Berlin, Germany 2011 – Travel grant awarded by Deutscher Akademischer Austausch Dienst (DAAD)